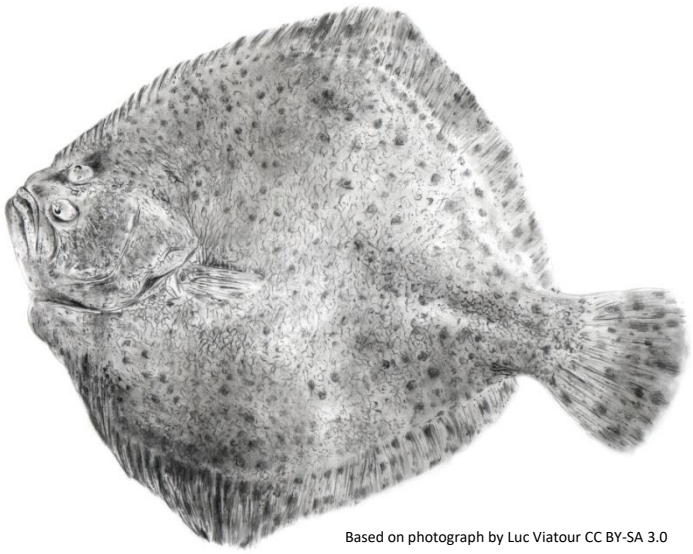
Chronic stress in fish

Investigation of the cellular response to persistent environmental hypercapnia and malnutrition in two marine fish species, turbot (Psetta maxima) and cod (Gadus morhua) via RT-qPCR



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"**It´s been a long, long journey**
Don´t know where I left my mind"

Allah-Las, long journey

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Summary

Stress in an unavoidable part of every organism's life. Consequently, the stress response, an individual's ability to counter harmful effects superimposed by its environment, has long been in the focus of scientific research. For finfish, this topic is not only relevant in the context of ecology but has also garnered lots of attention with respect to animal welfare in aquaculture. Yet, the stress response is an immensely complex process which involves numerous interconnected elements and which is influenced by multiple factors. In particular, the response to chronic stress still is not well understood and raises many questions regarding regulatory dynamics and mechanisms.

In this thesis, I investigated the impact of chronic stress in two economical important fish species, turbot (*Psetta maxima*) and Atlantic cod (*Gadus morhua*). Experiments with turbot were designed to reflect challenges relevant for aquaculture (malnutrition and severe hypercapnia), while cod larvae were exposed to conditions which simulated predicted future acidification of the oceans. In order to examine the impact of these stressors, I applied relative gene expression analyses via RT-qPCR. Transcriptional adjustments are considered a fundamental step in adapting to a persistent stressor and further reflect biochemical changes within the cellular milieu, which in turn are the foundation of stress-induced changes on higher levels of biological organisation. The aim of this study was to gain new insights in the respective coping mechanisms and detect potential communalities. Further, results were also screened for putative biomarkers for the detection and monitoring of environmental and culture induced stress.

The results demonstrated several changes in transcript abundances even under mild chronic stress. In each experiment, data pointed to divergent changes in gene expression between treatment levels, indicating different coping strategies dependent on the respective stress intensity. Further, my findings implied overall changes in lipid- and fatty acid metabolism as general part of the cellular response to chronic stress and that disturbances in these pathways may contribute to the formation of pathologies. Hints for additional hypoxia-like effects, reduced metabolic activity and increasing oxidative stress varied between the experiments, but seemed to increase parallel to the severity of the stressor. Several cues suggested that especially reactive oxygen species may play a central role in mediating both,

regulation of gene expression and detrimental consequences of chronic stress.

These findings provide new insights in the cellular response to chronic stress in fish and could help to improve contemporary stress and welfare concepts. Especially the impact of stress intensity-dependent intracellular ROS concentrations is not well documented for persistent challenges. Yet, the regulatory and damaging effects of reactive oxygen species provide a mechanistic explanation for divergent gene expression patterns and may also explain wear and tear like effects, which can occur even under mild stress. Finally, I was able to identify multiple genes, which can be utilized as stress biomarkers in future studies.

Deutsche Zusammenfassung

Stress ist ein unausweichlicher Bestandteil im Leben eines jeden Organismus. Dementsprechend ist die Stressantwort, also die Fähigkeit eines Individuums schädlichen Umwelteinflüssen zu begegnen, seit langem im Fokus wissenschaftlicher Forschung. Für Fische ist dieses Thema nicht nur im ökologischen Kontext, sondern auch in Hinblick auf artgerechte Tierhaltung in Aquakultur und Aquaristik von Bedeutung. Die Stressantwort ist jedoch ein ungemein komplexer Prozess, der sich aus zahlreichen, ineinandergreifenden Elementen zusammensetzt und von diversen Faktoren beeinflusst wird. Insbesondere die Reaktion auf chronischen Stress ist noch immer nicht hinreichend verstanden und wirft viele Fragen bezüglich der Regulation und den zugrundeliegenden Mechanismen auf.

In dieser Arbeit untersuchte ich den Einfluss von chronischem Stress auf zwei ökonomisch wertvolle Fischarten, den Steinbutt (*Psetta maxima*) und den Dorsch (*Gadus morhua*). Während ich mit Steinbutt Aquakultur-typische Stressoren testete (Fehlernährung und extreme Hyperkapnie), wurden Dorsch-Larven Bedingungen ausgesetzt, welche die zukünftige Versauerung der Ozeane simulierten. Zur Untersuchung der Auswirkungen dieser Stressoren verwendete ich relative Genexpressionsanalysen mittels RT-qPCR. Änderungen des Transkriptoms gelten nicht nur als essentieller Schritt in der Anpassung an anhaltenden Stress, sondern reflektieren auch biochemische Änderungen im zellulären Milieu, welche wiederum die Grundlage für stressbedingte Beeinträchtigungen auf höheren Ebenen biologischer Organisation darstellen. Ziel war es, neue Einblicke in die entsprechenden Bewältigungsstrategien zu erhalten und mögliche Gemeinsamkeiten zwischen diesen aufzudecken. Zudem wurden Resultate der Genexpressionsanalysen auch auf mögliche Stress-Biomarker hin untersucht.

Die Ergebnisse zeigten selbst unter mildem chronischen Stress Änderungen im Expressionsniveau mehrerer Gene. Dabei stellten sich divergierende Genexpressionsmuster zwischen den Behandlungsstufen als Experiment-übergreifendes Muster heraus, was auf unterschiedliche Bewältigungsstrategien in Abhängigkeit der entsprechenden Stress-Intensität hinwies. Meine Ergebnisse implizieren des Weiteren, dass Modulationen des Lipid- und Fettsäure-Stoffwechsels allgemeiner Bestandteil der zellulären Antwort auf chronischen Stress sind und Störungen in diesen Stoffwechselwegen zur Ausbildung von pathologischen

Veränderungen beitragen können. Weitere Hinweise auf hypoxieähnliche Effekte, reduzierte metabolische Aktivität und zunehmenden oxidativen Stress variierten zwischen den Experimenten, schienen jedoch mit steigender Intensität des Stressors zuzunehmen. Mehrere Indizien sprachen dafür, dass vor allem reaktive Sauerstoffspezies (ROS) eine zentrale Rolle spielen, sowohl in der Regulation der Genexpression als auch beim Hervorrufen der schädlichen Folgen von chronischem Stress.

Diese Funde erlauben neue Erkenntnisse bezüglich der zellulären Antwort auf chronischen Stress bei Fischen und könnten bei der Verbesserung aktueller Stress- und Tierhaltungskonzepte helfen. Insbesondere die Auswirkungen von Stressintensität-abhängigen intrazellulären ROS Konzentrationen sind für anhaltende Belastungen noch nicht gut belegt. Die regulatorischen und schädigenden Eigenschaften reaktiver Sauerstoffspezies liefern dabei sowohl eine mechanistische Erklärung für divergierende Genexpressionsmuster als auch für Abnutzungserscheinungen, die selbst bei milderem Stress auftreten können. Außerdem konnte ich mehrere mögliche Gene identifizieren, die sich zum Einsatz als Stressbiomarker in zukünftigen Studien eignen könnten.

INTRODUCTION

Stress and the stress response

Stress is an important event an organism encounters countless times throughout its life. The ability to cope under suboptimal environmental conditions is considered an adaptive process and essential for the maximization of both, fitness and survival. The importance of stress for finfish, as well as the response to it, has long been recognized as a topic of extensive importance in various fields of biology and veterinary medicine (Barton 2011). While, to this date, a consensus definition could not be established, a classic, often applied physiology-based approach defines the stress response as "the response of an organism to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting stage" (Barton 2011, Selye 1973). Thereby, stress can arise from anticipatory challenges, such as certain seasonal or life history events (e.g. breeding), as well as from unpredictable, stochastic effects.

In fish, the stress response is not only relevant within the context of ecology and evolution but also with regard to a sustainable aquaculture (Toni et al. 2018, Yildiz et al. 2017, Iwama et al. 2011). In the latter case, technical shortcomings or limitations in the realisation of an optimal rearing facility frequently prevent the provision of suitable, organism specific surroundings (Toni et al. 2018, Yildiz et al. 2017, Segner et al. 2012, Tacon et al. 2010, Colt 2006*).* Thereby, stress often cannot be avoided due to spatial constraints imposed on each individual. This inability to escape unfavourable environmental conditions in a fish tank, pond or cage render chronic stress as a crucial problem in aquaculture. However, persistent challenges can also arise in the wild, for example as the result of ecological upheavals, or in a laboratory setting.

Chronic sub-lethal stress can greatly affect an individual´s performance, including growth, development and reproduction, which in turn negatively influences biomass accumulation and population sizes (Schreck 2010, Barton 2002, Schreck et al. 2001, Pankhurst & Van der Graak 1997). Additionally, ongoing exposure to a stressor eventually exhausts the organisms coping capacities, resulting in the development of pathologies and other detrimental outcomes (Barton 2002). The avoidance of these maladaptive effects is of special importance in aquaculture: first, to maximize

production value and assure product quality and second, to meet the standards of animal welfare concerns (Poli 2009, Ashley 2007, Conte 2004). These concerns, which emerged over the last decades, put high emphasis on the animals' wellbeing. Confining to a functional approach to animal welfare, in which considerations regarding psychological or behavioural aspects are neglected, indicators of good welfare comprise the absence of disease and pathologies, as well as the ability to maintain homeostasis (Segner et al. 2012). The demand to provide suitable, species-dependent conditions and protect cultured animals from stress was also included in a specific set of guidelines, the so called "five freedoms" (FAWC, 1996).

Both, the desire to make general assumptions regarding the impact of potentially harmful agents on a fish species, as well as to survey welfare standards, created a need to assess environmental conditions and their ability to inflict stress in an individual. Thus, there is great interest in the development of suitable indicators, which provide information regarding the animal's condition and, therefore, allow to test whether individuals are able to adapt to a deteriorated surrounding (Müller-Graf et al. 2012, Segner et al. 2012, Håstein et al. 2005). This also includes the question whether the chronic exposure to a certain environment can negatively affect an individual without visible adumbrations, for example by diminishing the resistance against other stressors or by evoking carry over effects, which manifest in a later life history stage (Barton 2002). However, this poses a problem: What classifies as a stress response and what separates a successful compensatory response from a failed one? Answering these questions is difficult due to the intricate nature of the stress response. Even when leaving behavioural and psychological aspects aside, the stress response in teleosts is a multilevel event, encompassing numerous factors which determine the situation-dependent phenotype. These factors comprise species and sex-dependent differences, developmental stage, life history and physical condition, social status, season, water composition and quality, as well as nature and duration of the stressor (Shreck et al. 2001, Bonga 1997). In addition, the contribution of these factors can vary between different levels of biological organisation: Fish respond to most insults with a physiological and cellular stress response. Yet, while both processes intertwine, they are often considered separately.

The physiological stress response

Despite the fact that many aspects and details of the stress response still need to be unravelled, an understanding of the general chain of events, which takes place in fish (and other higher organisms) has been established: In an attempt to characterize the different stages of the stress response over a time course, also with regard to varying levels of biological organisation, scientists often distinguish between the primary, secondary and tertiary stress response (Barton 2002, Iwama 1998). With the onset of stress, the primary response provokes the release of several stress hormones into the blood stream. During the secondary response, various physiological and cellular changes occur, for example, shifts in metabolism, respiration or immune functions. Finally, the tertiary response relates to changes in the overall performance traits, such as growth, development or behaviour. This progression demonstrates the supposed connection between physiological and cellular stress response: Modifications on the cellular and sub-cellular level are expected to be the outcome of preceding shifts in endocrine activity. Biochemical and morphological changes in the cell, in turn, are the basis for changes in physiology and performance, as well as for the development of potential pathologies.

Essential to the primary response is the hypothalamic-pituitary-interrenal axis (HPI), which segregates glucocorticoids, such as cortisol (Bonga 1997). These hormones trigger the mobilization of energy depots and are central mediators of the adaptation process. At the same time, incessantly elevated cortisol concentrations are associated with reduced growth rates, delayed reproduction and other maladaptive outcomes of chronic stress (Schreck et al., 2001, Mommsen et al. 1999). Thus, these hormones have been applied as stress indicators in numerous studies, especially within the context of animal welfare in aquaculture (Ellis et al. 2012).

In fish, however, various studies reported an absence of a correlation between cortisol levels and growth rates, which raised concerns regarding the utility of hormones as stress indicators during periods of persistent stress (Van Weerd & Komen 1998). Indeed, in the human model, cellular resistance to cortisol as a result of the progressive down-regulation of glucocorticoid receptors in the face of ongoing stress has been repeatedly observed (for example in Miller et al. 2008, Pariante 2006, Miller et al. 2002), and it is likely that similar phenomena occur in teleosts. Consequently, measurements of cortisol concentration may become increasingly

unreliable when a reduction or alteration in receptor abundances prevents to deduce subsequent cellular effects. Moreover, interactions of cortisol with other mediators of the regulatory network may result in more complex relationships. Thus, it is questionable to which extent blood cortisol concentrations are useful stress indicators when it comes to chronic stress.

Despite this drawback, stress hormones have also been considered in the development of stress models, which aim to provide explanatory frameworks for the time- and intensity-dependent impact of stress onto an organism. One of the most popular models, which also incorporates the mediating role of cortisol and other glucocorticoids in the adjustment to social and environmental stress, refers to "allostasis" (McEwen & Wingfield 2003, 2010): The concept of allostasis is best described as "stability through change", referring to consecutive behavioural, physiological and biochemical adjustments of an organism in order to maintain homeostasis. In this regard, homeostasis is defined as the few set points that are truly essential for life, as for example oxygen tension or pH-value. The model contrasts the energy available for the organism with the energy that is required to fuel daily routines as well as unexpected, stressful events. The cumulated energy required to keep all currently necessary biological processes running is defined as the "allostatic load" (McEwen & Wingfield 2003). If allostatic load surpasses the energy available from the environment and possible energy stores, the energy balance becomes negative and results into a so-called emergency life history stage (ELS) for the organism. This ELS is characterized by rapid physiological and behavioural changes in order to exalt the chance to survive under the given circumstances. If the ELS fails to reduce allostatic load to a sufficient degree, pathological effects and death can occur. There have also been attempts to use the allostatic model in order to specify a functional welfare definition (Segner et al. 2012, Korte et al. 2007).

Yet, the allostatic model has limitations, which not only arise from the use of energy as the central measuring unit, but also from the appropriation of glucocorticoids as an indication of energy expenditure (see Romero et al. 2009). In particular, the assumed linear relationship between stress intensity, glucocorticoid levels and, therefore, energy expenditure may only be valid during the early phase of the stress response (Mommsen et al. 1999). This leads directly to the next problem, as the question of what exactly causes allostatic load is not yet sufficiently answered (McEwen & Wingfield 2010). Consequently, implementation of several factors which

contribute to individual variation, for instance epigenetics, can be difficult. This can also account for dietary aspects, as for example malnutrition.

Another example for a non-linear relationship between stress intensity and its impact on the organism can be observed during mild stress, which may even be advantageous for the individual: This phenomenon, in which the exposure to low concentrations of an otherwise harmful stressor exerts a beneficial adaptive response is labelled as “hormesis “ (Calabrese 2008, Mattson 2008, Calabrese & Baldwin 2002). Hormesis is assumed to reflect an anticipatory effect in which mild stress triggers protective mechanisms in preparation for a future aggravation, which, as a side effect, may also enhance the organism’s resistance against other stressors. The hormetic response curve has been described with consistent characteristics for a vast range of mediators, including stress hormones, highlighting the fundamental and widespread nature of this process (Calabrese & Blain 2005). Thereby, the consistency in width and magnitude of the response curves make the hormetic principal highly generalizable (Calabrese et al. 2013, Calabrese 2008). However, as the assessment of hormetic dynamics over a period requires the use of multiple different doses and sampling points, knowledge regarding hormesis under conditions of chronic mild stress are still scarce (Calabrese & Baldwin, 2002).

Both, the limitations of allostasis and hormesis as well as the declining informational value of cortisol and other glucocorticoids over time highlight the problems which must be considered when investigating chronic stress: In comparison to the early phase stress response, other mechanisms and mediators may become relevant if the stressor persists. Thus, stress biomarkers, which have been established for the acute stress phase, may not be reliable in later stages of the stress response. On the molecular level, the contingent progressive insensitivity towards external signals suggests that the cell may become increasingly regulated by internal processes. Also, as pathological effects, diseases or a general diminished performance capacity originate on a cellular level, it may be worthwhile to apply molecular methods when investigating chronic stress.

The cellular stress response

The understanding of the cellular stress response, including associated changes in the regulation of biochemical pathways, is vital to answer questions

concerning environmental stress, animal welfare and the identification and evaluation of suitable biomarkers. It is known that cellular adjustment mechanisms can be separated into two major processes, both of which occur simultaneously: the cellular homeostatic response (CHR) and the cellular stress response (CSR) (Kültz 2005). The CHR refers to mechanisms that are activated in order to counter and restore threats to the cellular homeostasis after one or more environmental parameters have changed. Comparable to the idea of allostasis, the cellular homeostatic response results in a permanent shift of the interior milieu until environmental variables change again. The CHR is therefore regulated by parameter-specific sensor molecules and corresponding signal-transduction pathways (Kültz 2005).

The cellular stress response, on the other hand, is triggered by macromolecular damage, which appears to be a general trait of stress. By reacting to structural and functional impairments of DNA, enzymes or lipids the cell can immediately initiate counter measures without the need to rely on stressor-specific signal transduction pathways. Additionally, the orchestration of repair and protection mechanisms can be achieved by a relatively small set of common regulators. One example for a group of molecules which appear to play a central role in the CSR is given by reactive oxygen species (ROS) and, to some extent, reactive nitrogen species (RNS). Both substance classes can be summarized as reactive molecule species (RMS). An increase in intracellular RMS concentrations seems to be an ubiquitous outcome of stress (Kültz 2005). Hence, reactive molecule species are not only considered to cause the damage which initiates the CSR, but also to function as crucial secondary messengers, as they influence the activities of several redox-sensitive pathways. Consequently, anti-oxidants, such as the enzymes of the glutathione-S-transferase superfamily (Hayes & McLellan 1999), can be appended to the CSR. Indeed, a large scale of microarray studies on gilthead sea bream (*Sparus aurata*) found genes related to ROS scavenging up-regulated during continuous confinement stress (Calduch-Giner et al. 2010). Additional ubiquitous characteristics of the chronic cellular stress response comprise changes in major metabolic pathways (Kültz 2005). These changes mainly seem to foster the mobilization of energy reserves, which are considered to fuel repair and compensation mechanisms, as well as the inhibition of anabolic processes.

Another family of enzymes which are generally associated with the CSR are the so-called heat shock proteins (HSPs) (Deane & Woo 2011, Basu et al. 2002, Feder

& Hofmann 1999). These molecules function as chaperones, i.e. they enclose other native proteins and, thus, assist these proteins during the process of folding as well as insulate them against disruptive influences. The gene family comprises various members with different molecular masses, most of which occur in inducible and constitutively expressed homologues. In fish, several HSPs seem to comprise additional functions, related to the immune system, apoptosis, inflammation, growth and development (Roberts et al. 2010).

However, Iwama et al. (2004) raised the question whether heat shock proteins are valuable stress indicators, specifically when investigating chronic stress, as changes in their expression seem to depend on the tested stressor and the species under investigation. In general, the molecular response to stress has revealed itself as an immensely complex process (Kültz 2005). Especially in comparison to the well-established physiological response, details regarding changes in the cellular biochemistry during a persistent challenge are still fragmentary and far from complete (Prunet et al. 2008). However, in order to identify viable stress biomarkers, a detailed understanding of the mechanisms, which are involved in the chronic stress response, as well as in the development of pathologies, is necessary. Advanced knowledge could further help to improve contemporary stress models and address questions related to ecology and animal welfare.

Gene expression analysis to investigate chronic stress

In this thesis, I assessed relative gene expression via real-time quantitative PCR in order to investigate chronic stress in marine fish species. Changes in transcriptional regulation constitute the basis of cellular adjustment processes and can also provide information regarding the capacity of the CSR at the time of measurement (Kültz 2005). Thus, gene expression analysis is a well-suited method to illuminate new aspects of the stress response, as well as to identify common response elements which may function as stress biomarkers (Prunet et al. 2008, Valasek & Repa 2005). When possible, I used the dynamic array gene expression approach, which combined the sensitivity of Real-Time qPCR with a relatively high throughput. Genes where chosen to cover "classical" stress markers, such as HSPs or anti-oxidants, but also to encompass a wider range of other cellular functions, for example inflammation, metabolism or the regulation of cellular processes.

Fish species and tested stressors were chosen to represent settings with relevance for aquaculture and ecology. As a representative of a highly valuable aquaculture species, juvenile turbot (*Psetta maxima*) was subjected to mild dietary stress (Chapter I) and severe environmental hypercapnia (Chapter II) for prolonged periods of time. Further, the impact of chronic hypercapnia was also investigated in cod larvae (*Gadus morhua*), a species which is widespread throughout the North Atlantic (Chapter III). In this case, however, carbon dioxide levels were set to concentrations which mirror predicted future ocean scenarios. Thereby, differences between the respective experimental settings, mainly in applied species, stressors and stress intensities, were chosen to identify potential common elements of the transcriptional response to chronic stress. This, in turn, may help to deduce general aspects of the CSR. Furthermore, in order to connect the findings to higher levels of biological organisation, available physiological or histological information were incorporated in the analyses, as well.

Therefore, the main aim of this thesis was to test in which way different experimental settings can affect the expression profile of tested target genes. The respective findings were meant to address the following questions: 1) Are there indications of mechanisms or pathways which may be general elements of the chronic cellular stress response? 2) Can these potential pathways be used to enhance contemporary stress models and, thus, further support the formulation of animal welfare standards? 3) Are there target genes which have the potential to be valuable biomarkers for chronic stress or, alternatively, for an increased susceptibility to stress?

Thesis outline

This thesis is subdivided into three chapters, each presented in the form of a manuscript. Thus, each chapter is structured into abstract, introduction, material and methods, results and a discussion. A brief summary of the respective attempts and chosen methods is presented below.

Chapter I

In the first experiment, I was interested in investigating the impact of chronic mild stress on the transcriptional adjustments within a primary coping organ. Therefore, I retrieved liver samples from juvenile turbot (*Psetta maxima*), fed with a diet, in which fish meal was substituted at different degrees of purified rapeseed protein concentrate (RPC) over a time course of twelve weeks. To assess potential effects of the contained antinutrients on the cell's biochemistry, I developed qPCR assays for 31 genes of interest (GOIs), based on the genetic information available for turbot in the NCBI data base at this time. Genes were selected to cover a broad spectrum of functions and metabolic pathways. In comparison with allocatable performance data, changes in gene expression were also contemplated in regard to potential implications for the welfare of fish and the utility of certain genes as stress biomarkers.

Chapter II

In the second experiment, I wanted to address the other end of the spectrum and look at chronic severe stress, close to, or even beyond the coping capacity of the cell. Thus, I collected gill samples of juvenile turbot continuously exposed to varying degrees of high carbon dioxide concentrations for eight weeks. To enhance the output of the experiment, the set of qPCR assays established in chapter I was further extended by additional GOIs, associated with stress and the immune response, to a total number of 40. Accessory histological information was used to compare potential effects across levels of biological organisation. As in the first chapter, while the focus was placed on the unravelling of underlying mechanisms involved in the cellular

adjustment to chronic stress, I also attempted to connect findings to contemporary stress and welfare concepts and further screened results for new potential stress biomarkers.

Chapter III

In the last study, I applied gene expression analysis to investigate chronic stress in whole cod larvae (*Gadus morhua*). The samples originated from an experiment in which the effects of ocean acidification-like conditions were tested during the developmental period from hatch to early juvenile life history stage. Larvae had been shown to respond to chronic environmental hypercapnia by delaying metamorphosis and developing lipid droplets throughout the body, thus, providing the opportunity to study a transcriptional response to stress which has resulted in maladaptive effects on higher levels of biological organization. Therefore, in cooperation with the Alfred Wegener Institute for Polar and Marine Research, a new set of Real-Time PCR primer assays was developed, in which target genes were selected to cover key metabolic processes. The main goal was to identify transcriptional shifts which may provide insides regarding the molecular basis for observed physiological repercussions.

CHAPTER I

Effects of dietary purified rapeseed protein concentrate on hepatic gene expression in juvenile turbot (*Psetta maxima*)

Abstract

Despite considerable progress in the production of alternative diets, small concentrations of antinutrients remain common in aquaculture nutrition, resulting in a perpetual limitation with regard to the inclusion of plant ingredients in aquafeeds. These compounds are known to impair the general performance of fish when fed for a prolonged period of time, potentially affecting the animal's susceptibility to stress, too. Therefore, a 12-week feeding trial was conducted to examine the chronic effects of purified rapeseed protein concentrate (RPC), containing low concentrations of glucosinolates and phytic acid, on the relative expression of multiple target genes in the liver of juvenile turbot (*Psetta maxima*, L.). Our results revealed divergent patterns of gene expression, suggesting different coping strategies dependent on the grade of RPC substitution. Data implied increased metabolic rate of turbot fed a 33% RPC-substituted diet due to an up-regulation of cytochrome c oxidase mRNA, accompanied by minor adjustments in metabolic pathways. While no signs of reduced welfare were found, data adumbrate a beneficial hormetic reaction. In the highest treatment level (66% RPC), diminished fish condition and reduced growth performance coincided with a down-regulation of insulin-like growth factor I, further indicating a potential impaired resistance to stress. An additional down-regulation of transferrin hints towards an increased liability to bacterial infections.

Introduction

In aquaculture, fishmeal is still considered to be the 'gold standard' in the feeding of fish due to its optimal protein composition. However, the constant growth of the aquaculture industry has created a need to find alternative nutritional sources due to highly fluctuating landings for the fishmeal industry over the last two decades accompanied by rising prices, which reached an all-time high of almost 2000 US$/t in

2013 (Tacon et al. 2010, Naylor et al. 2009, Tacon & Metian 2008). Much research effort was devoted to evaluate the use of plant proteins as substitutes in the nutrition of marine and freshwater fish (Gatlin et al. 2007). Due to its beneficial protein profile, very similar to that of fishmeal, rapeseed (*Brassica napus*) emerged to be one of the most promising candidates (Enami 2011).

Several studies in rainbow trout (*Oncorhynchus mykiss*, Hernandez et al. 2013, Slawski et al. 2012) and Atlantic salmon (*Salmo salar*, Liland et al. 2013) reported a threshold percentage of rapeseed-derived protein sources in the diet of different fish species, above which negative effects on growth performance were observed, such as lowered feed intake (FI), elevated feed conversion ratio (FCR), lowered protein conversion ratio (PER) or lower specific growth rates (SGR). Antinutritional factors, included in the plant-derived fraction of the diet, are considered the main agents causing the observed adverse effects.

The main antinutrients found in canola/rapeseed are glucosinolates (GLS), phytic acids (PAs), sinapinic acid and tannins. While glucosinolates themselves are biologically inert, their degradation products result in reduced food intake via impaired palatability, decreased individual growth and, therefore, diminished aquaculture production (Tripathi & Mishra 2007). Phytic acids aggravate the access of digestive enzymes to several nutrients by chelating them in complex structures. Consequently, they reduce the bioavailability of important dietary components such as phosphor, cations (e.g. iron, calcium or copper) and positively charged proteins (Francis et al. 2001), hence, potentially inflicting malnutrition (Mawson et al. 1994). Likewise, tannins interfere with the digestive processes by binding to feed components such as proteins or minerals or to digestive enzymes, directly.

Several technical improvements allow the production of more purified rapeseed products containing less total antinutrients (Drew et al. 2007). Nagel et al. (2012) tested the effect of rapeseed isolate (RSI) as a fishmeal substitute in the diet of juvenile turbot (*Psetta maxima*), a high-value marine aquaculture species. Turbot showed a reduced FI and FCR, alongside a reduced growth performance at a substitution level of 66% and above. Further, body composition was altered towards reduced dry matter and crude proteins. Moreover, the authors observed severe hepatic hypertrophy in fish fed a diet in which 33% of fish meal was replaced with RSI. While not identifying pathologies per se, these findings demonstrate that even highly purified rapeseed products still inflict adverse effects on turbot - directly, by

interacting with other nutrients in the diet and the digestive system, as well as indirectly by affecting the palatability and, hence, food intake. Consequently, juvenile turbot may become more prone to stress.

In this study, we investigated gene expression in livers of juvenile turbot subjected to a 12-week feeding trial, in which fishmeal was partially substituted with rapeseed protein concentrate (RPC), 33% (R33) and 66% (R66), respectively. The liver is an essential organ and exhibits regulatory function of general metabolism, digestion and, to some extent, immune defence, making it an obvious target for the investigation of dietary-mediated effects. We analysed transcription rates of 31 genes, representing several important cellular pathways and processes. Our main goal was to illuminate underlying molecular mechanisms involved in the metabolic adjustments to the experimental diets. Further, we screened gene expression patterns for indications of malnutrition or increased susceptibility to stress, with the goal to develop biomarkers for fish nutritional conditions that are more sensitive than phenotypic traits such as growth rates alone.

Material and methods

Experimental Setup

Three isonitrogenous (587 g CP kg^{-1} ± 1.5) and isocaloric (22.2 ± 0.51 MJ kg^{-1}) experimental diets were formulated to conduct a 12-week feeding trial with juvenile turbot (Psetta maxima). The control group was fed a pure fishmeal-based diet (R0), whereas treatment groups received pellets in which fishmeal was partially substituted with 33% (R33) and 66% (R66) RPC, respectively. Dietary formulation, nutrient composition, amino acid profiles and antinutritional factors are summarized in Table 1.1 (see also Slawski et al. 2011). Juvenile turbot were obtained from Ejsing Seafarm (Vinderup, DK) and separated into nine tanks (fourteen fish per tank, three replicates per treatment level), each containing 94 L of artificial sea water. Fish were reared at constant water parameters (temperature: 17.1 ± 1.1 °C; O_2: 7.8 ± 0.2 mg L^{-1}; salinity: 25.8 ± 0.5 g L^{-1}; pH > 7.8; NH_4^+: <0.07 mg L^{-1}; NO_2 : <0.13 mg L^{-1}). Prior to the experiment, animals were fed R0 for 2 weeks and subsequently starved for 2 days. During the feeding trial, fish were fed once a day to apparent saturation.

Table 1.1: (a) Formulation of the diets (g kg $^{-1}$). (b) Dietary nutrient and amino acid composition. (c) Nutritional composition of the fish meal and rapeseed concentrate.

(a) Food Component	R0	R33	R66
Fish meal	450	300	150
Rapeseed protein concentrate	0	145	295
Soy protein concentrate	100	100	100
Shrimp meal	85	85	85
Blood meal	95	95	95
Starch	90	90	90
Dextrose	75	70	60
Fish oil	55	65	75
Vitamin/Mineral mix[1)]	20	20	20
Wheat gluten	30	30	30

(b) Ingredient	R0	R33	R66
Nutritional composition (% of dry matter)			
Crude protein	57.5	57.7	59.1
Crude lipid	10.8	11.1	10.8
Ash	13.6	12.3	10.8
Phosphor	1.7	1.4	1.1
Crude fibre	0.8	0.7	0.5
NfEa[2)]	17.4	18.2	18.7
Gross energy[3)] (MJ kg^{-1})	21.2	21.6	21.8
Amino acid composition (g kg^{-1} crude protein)			
Arginine	59.7	61.3	59.1
Histidine	24.7	31.5	31.6
Isoleucine	31.5	32.3	30.7
Leucine	79.4	82.5	79.3
Lysine	58.1	62.1	59.9
Methionine & Cysteine	23.9	26.6	28.4
Phenylalanine	43.7	44.9	42.3
Threonine	37.5	38.5	36.6
Valine	51.9	53.4	50.5
Antinutritional Factors			
Glucosinolates (µmol g^{-1})	0	0.2	0.4
Phytic acid (g Kg^{-1})	0	2.5	5.2

Table 1.1 (continued)

(c) Ingredient	Fish Meal	Rapeseed Concentrate
Crude water (% of air-dried substance)	8.4	5.6
Nutrient composition (% of dry matter)		
Crude protein	69.0	71.2
Crude fat	7.0	0.6
Crude ash	20.7	16.1
Phosphorous	2.9	0.9
Crude fibre	0.5	0.5
NfE[2)]	2.9	11.6
Gross Energy[3)] (MJ kg^{-1})	19.9	19.4
Amino acid composition (% of dry matter)		
Arginine	4.03	4.83
Histidine	1.38	2.75
Isoleucine	2.50	2.70
Leucine	4.45	5.39
Lysin	4.52	5.60
Methionine + Cysteine	2.19	4.64
Phenylalanine	2.43	2.56
Threonine	2.69	2.97
Valine	3.07	3.67
Antinutritional factors		
Glucosinolates (µmol g^{-1})		1.32
Phytic Acid (g $100g^{-1}$)		1.77

[1)] aAA-Mix 507101, Vitfoss, Gråsten, Denmark.
[2)] N-free extra compounds = 100 - (%crude protein + %crude Lipid + %ash+ %crude fibre).
[3)] calculated: Crude protein = 23.9 MJ kg^{-1}; Crude lipid = 39.8 MJ kg^{-1}; Crude fibre = 17.6 MJ kg^{-1}.

At the end of the experiment, six fish were randomly chosen per tank from each treatment group (total of eighteen fish per group) and sacrificed. Weight and length were recorded, and liver from each individual was dissected and preserved in RNAlater (Qiagen, Hilden, Germany) for later gene expression analyses.

RNA extraction. quality assessment and cDNA synthesis

Total RNA was extracted using the Invitrap Spin Tissue RNA Mini Kit (Stratec, Berlin, Germany) according to the manufacturers' instructions. To ensure RNA integrity, a representative number of samples (50%) were analysed with the Experion System (Bio-Rad, Munich, Germany). A RNA quality indicator of 8.0 or higher was defined as a minimum value a sample had to meet to be considered for further downstream applications. RNA concentrations were determined via the QuBit® 2.0 Fluorometer (Life technologies, Carlsbad, CA, USA). Per sample and tissue type, 700 ng of total RNA was used for reverse transcription (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany). This procedure included the elimination of genomic DNA prior to the actual synthesis of cDNA. An aliquot of gDNA digested RNA was prepared for each sample to serve as a RT-control in the subsequent gene expression analysis. The manufacturer protocol for cDNA synthesis was changed as follows: the gDNA wipe-out step was elongated to 5 min and reverse transcription was conducted for 20 min instead of fifteen. Both changes improved cDNA synthesis.

Primer design and gene expression analysis

Sequences for 32 genes of interest were obtained from GenBank (http://www.ncbi.nlm.nih.gov) via key word search. Genes were selected to cover a broad range of physiological functions. The whole gene set comprised representatives of important pathways, such as cellular stress response, inflammation, glycolysis, somatic growth or regulation of apoptosis (Table 1.2). Gene-specific primer pairs were designed using the software PRIMER3 (Untergasser et al. 2012, Koressaar & Remm 2007). Different dilution series ranging from 1 : 2 to 1 : 6250 cDNA dilution were created to evaluate efficiency, sensitivity and specificity of each pair of primer.

The associated tests were conducted in duplicates on a StepOne Thermo-Cycler (Applied Biosystems, Foster City, CA, USA) for each gene. Primer specificities were tested via melt curve analysis. The subsequent gene expression analysis of the experiment was performed in triplicates for each gene-sample combination on the BioMark® HD System (Fluidigm, San Francisco, CA, USA), applying 96 x 96 Dynamic Array gene expression chips. Samples were randomly distributed across two chips, thereby placing three samples on both chips to function as inter-run calibrators.

Table 1.2: PCR primer sequences, accession numbers and calculated efficiency.

Gene	Abbr.	Accession No	Primer Sequence	Eff%
HMG-CoA reductase	HMG-CoAr	JN542428.1	fw: TGCTGGAGTTGTCCCGTGAGAGT	108.6
			rev: CTGTGGGCGTGAACCATCACCAA	
Adenylat kinase 2	ak2	DQ848978.1	fw: ACGACTTGCTGGACAAGAGACGC	100.8
			rev: GCGGCCACTGGGCTGATGAATTA	
Akirin	Akirin	HM237345.1	fw: GACCATGAGGAGAAGATCCGGGAGG	107.5
			rev: TCAGGAGACATAACTAGCAGGCCGG	
Alpha tubulin	TubA	DQ848853.1	fw: GCCCTACCCTCGTATCCACT	100.1
			rev: TGATGTCAGCCACAGAGAGC	
Apolipoprotein E	apoE	AJ236883.1	fw: AGGCCACCGCTAAGGAGCTTTTCA	102.5
			rev: TTCCCAACCTGCTCTTGGATCTGGG	
Arginase type II	Arginase	AF467774.1	fw: ACGCGGACATAAACACGCCCA	99.82
			rev: TGGCTGGCATCTTGTCTTGGAGC	
Cathepsin D	CathD	EU077233.1	fw: TCGCCTGCTTGCTTCACCACA	107.9
			rev: GCCCGACAAACTGCCAGATCCAT	
Cell death-regulatory protein	GRIM19	FJ617007.1	fw: TCCAGGTTGGAAGGTCGGTGAGA	105.3
			rev: CGTACCACAAGAAGCCAAAACGCT	
Cold-shock domain	CSD	DQ840135.1	fw: ACGGCGAGGAGGACAAGGAGAAT	103.6
			rev: TTGAAGTTGCGGCGGTAGCGA	
Copper chaperone	CCS	DQ400684.1	fw: AACCCGCAGTTTTCGACACGTCA	104.9
			rev: TGAACAGCCCTCGCACGTCATC	
Cytochrome P4501A	Cyt450	AJ310694.1	fw: ATCGCTCTCCTCTTCTCTCT	99.41
			rev: TTAGAGGTGCAGTGTGGAAT	
Cytochrome-C-oxidase subunit 1	COX	DQ848855.1	fw: TCGCGCTTACTTCACCTCCGCTA	99.92
			rev: CATGGAGTGTTGCGAGCCAACTGA	
Elongation factor 1 alpha	EF1α	AF467776.1	fw: ACCGAGGTGAAGTCTGTGGA	102.3
			rev: CGGACACGTTCTTGATGTTG	
Glutathione S-transferase	gst	DQ848966.1	fw: GGGTTCGCATCGCTT TT	89.88
			rev: GGCCTGGTCTCGTCTATGTACT	
Glycerinaldehyde-3-phosphate dehydrogenase	GAPDH	DQ848904.1	fw: CAGTGTATGAAGCCAGCAGAG	111.3
			rev: ACCCTGGATGTGAGAGGAG	
Heat shock protein 70kDa	HSP70	EF191027.1	fw: CCGCTGCTGCTATTGCCTATGGT	100.5
			rev: TGCCGCCACCGAGATCAAAGATG	
Heat shock protein 90kDa	HSP90	EU099575.1	fw: ATCAACAACCTGGGAACCATCGCC	103.3
			rev: GAAACCCACGCCAAACTGACCGA	
Insulin-like growth factor 1	IGF1	FJ160587.1	fw: TGTACTGTGCGCCTGCCAAGACTA	102.6
			rev: TGCTGTGCTGTCCTACGCTCTGT	
Interferone regulatory factor 5	IRF5	JF913460.1	fw: TCCGAACCCTCCGCCTTTTGAGA	99.6
			rev: TACGGGAACCACCTGGACGATGA	
Interleukin-1 beta	IL1	AJ295836.2	fw: ACCAGACCTTCAGCATCCAGCGT	96
			rev: TTCAGTGCCCCATTCCACCTTCCA	

Table 1.2 (Continued)

Lipoprotein lipase	LPL	JQ690822.1	fw: TCCCTTTGTTATGCCTGTCC	93.36
			rev: GCTGATGATTGAGTCCTTCTCC	
Low molecular weight polypeptide	LMP7	FJ617008.1	fw: GGGCATAGTTCACGCTACGCACA	89.85
			rev: CGTGTGTCGGGGTCAAACTCAGAA	
Lysocyme C	LysC	EU747734.1	fw: GAACGCTGTGAATTGGCCCGACT	86.63
			rev: GTTGGTGGCTCTGGTGTTGTAGCTC	
Natural resistance-associated macrophage protein	NRAMP	EU747732.1	fw: CGTCTTTGTGGTGGCTGTCTTTGCT	104
			rev: TCCCGGTTGCGTTGCATTGCT	
Polyunsaturated fatty acid elongase	ELO	AF465520.2	fw: CGTTCCTCCACATTTACCACCACGC	105
			rev: TTGAGGGATGCGCCGAAGTACGA	
Protein phosphatase 1 catalytic subunit beta	PP1bc	DQ364569.2	fw: GCGTGGACGAAACTCTGATGTGCT	97
			rev: ATCGCTTCTTTGGAGCTTGGGCTG	
Signal transductor and activator of transcription 2	STAT2	FJ719015.1	fw: CGGCAGCAGAAAACCTGCATTGG	106.8
			rev: TCCCGCACCTGGAACAAACACAC	
Toll-like receptor 3	Tlr3	FJ009111.1	fw: GACGTGCTGATCCTGGTCTTTCTGG	106
			rev: AGCTCAGGTAGGTCCGCTTGTTCA	
Transferrin	Tf	AJ277079.1	fw: ATCGTTGGAGGAGGCGGTAAGCA	99.34
			rev: TGGGTCTTGGAGCAGTCCCCTTT	
Tumor necrosis factor alpha*	TNF	FJ654645.1	fw: AAAAGAAGTCGGCTACGGGGTGGA	93
			rev: TTCCAGTGCCAAGCAAAGAGCAGG	
Δ6-Fatty acid Desaturase*	DFAD	AY546094.1	fw: TGGAGAGTCACTGGTTTGTGTGGGT	91.5
			rev: AGGTGGCCTGTAGCTGCATGGTTA	

*Efficiency value derived from dilution series conducted on StepOne cycler (see text)

To control for contaminations via undigested gDNA, each chip contained three non-template controls as well as three RT-controls. Also, to account for putative variations in the amplification efficiency of gene assays between the StepOne Cycler and the BioMark® HD System, two 1 : 5 stepwise dilution series (ranging from a 1 : 10 to a 1 : 1250 dilution) were included, each created from a different sample.

Data processing and data normalization

The data output from the BioMark® HD System was pre-analysed with the associated real-time PCR analysis software (Fluidigm). Replicates were visually controlled via melt curve analysis to assure only one major PCR product.

Subsequently, the software QBASE (Biogazelle, Zwijnaarde, Belgium) was applied for further data processing, normalization and calculation of calibrated normalized relative quantities (CNRQs). Replicates were screened for outliers and high standard deviations. Triplicates yielding a standard deviation > 0.5 (0.3 in case of

duplicates) were excluded from further analyses. Incorporating the whole set in the analysis, the GeNorm algorithm, implemented in QBASE, identified GRIM19, PPbc1 and CDS as the best combination of reference genes (Vandesompele et al. 2002). Normalized relative quantities (NRQ) were calculated according to Hellemans et al. (2007), incorporating gene specific amplification efficiencies and normalization to multiple reference genes:

$$NRQ = \frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[f]{\prod_0^f E_{ref_0}^{\Delta Ct,ref_0}}}$$

The average crossing point across all samples for a given gene was applied as a calibrator. Afterwards, the software converts NRQs into CNRQs by correcting for inter-run variation.

Statistical analysis

The software R was employed for statistical analyses (R Core Team 2012). CNRQ values were checked for normal distribution (Shapiro–Wilk test) and extreme values by visualizing via box-plots, QQ-plots and histograms, gene by gene. If necessary, values were transformed (log or square root) to match criteria for parametric tests. To evaluate the suitability of data for cluster analysis, correlation patterns were examined via Bartlett's test of sphericity, as well as the Kaiser–Meyer–Olkin measure (KMO) of sampling adequacy (Field et al. 2012). The KMO values range from zero to one and compare the correlation between variables with the partial correlation between variables, hence, indicating whether correlation patterns are more diffuse (close to zero) or more compact (close to one). In general, KMO values of 0.5 or better are considered necessary to indicate the suitability of a variable for factor analysis. To test for dissimilarity between the treatment groups, a between class analysis (BCA) was conducted. A multivariate analysis of variance (MANOVA) was applied to test for an overall significant treatment effect. Subsequently, ANOVAs were conducted to identify genes differently expressed between the groups. As post hoc exploration of differences in detail, Tukey tests were conducted.

Weight and length data of the juvenile turbot were used to calculate Fulton's condition index. (Bolger & Connolly 1989), with K = body weight (g)/total length (cm)^3

x 100. A Kruskal–Wallis test was used to test for differences in mean animal condition between the groups.

Results

Animal condition

Fish fed with a diet of fishmeal substitution level of 66% (R66) were in an inferior condition compared to R33 and the control group. Important physiological parameters, for example growth rates, were inferred from animals other than those selected for gene expression analysis and published elsewhere (Slawski et al. 2011). Physiological data are summarized in Table 1.3.

Table 1.3: Physiological data as reported by Slawski et al. (2011). Subjected to the same experiment, K* represents the condition calculated for phenotypes investigated in this study.

	R0	R33	R66
Start weight (g fish^{-1})	73,1 ± 1,0	74,1 ± 0,4	73,3 ± 0,4
Final Weight (g fish^{-1})	147,5^{a} ± 10,3	145,0^{a} ± 7,5	122,2^{b} ± 4,5
SGR (% day^{-1})	0,83^{a} ± 0,07	0,8^{a} ± 0,07	0,61^{b} ± 0,05
Food Intake (g Fish^{-1})	73,9^{a} ± 8,2	82,6^{a} ± 10,8	58,2^{b} ± 1,1
FCR (g g^{-1})	1,00^{a} ± 0,06	1,16^{b} ± 0,03	1,20^{b} ± 0,09
PER	1,75^{a} ± 0,11	1,49^{b} ± 0,04	1,42^{b} ± 0,11
Survival Rate	94,4 ± 9,6	100 ± 0,0	100 ± 0,0
K	1,80^{a} ± 0,16	1,76^{a} ± 0,12	1,56^{b} ± 0,13
K*	2,04^{a} ± 0,38	2,05^{a} ± 0,18	1,89^{b} ± 0,1

Primer assay efficiency

Efficiency values derived from the dilution series incorporated on the 96 X 96 Dynamic Array gene expression chips were applied for the calculation of the CNRQs because the corresponding dilution steps underwent the same treatment as the sample replicates and, therefore, are more accurate. For TNF and DFAD, the samples chosen for dilution series failed to provide enough data for a satisfying efficiency calculation. This was due to an unlucky combination of technical failure (air bubbles, which prevented Cp evaluation in some replicates) and low copy number (indicated

by an already high Cp in the undiluted step). Therefore, the amplification efficiency based upon the tests performed on the StepOne Cycler was used for further calculations.

Overall group comparison

The Kaiser–Meyer–Olkin measure was applied to evaluate the suitability of each gene for a factor analysis. Three genes (ApoE, EF1a and IL1) yielded too low KMO values and were thus excluded from BCA. Overall, KMO of the remaining genes was 0.72. Bartlett's test of sphericity yielded a chi-square value of 976.3 ($P < 0.0001$), confirming sufficient correlations between the genes relative expression values for cluster analysis. The BCA, applying the diet as factor, indicated overall dissimilarity

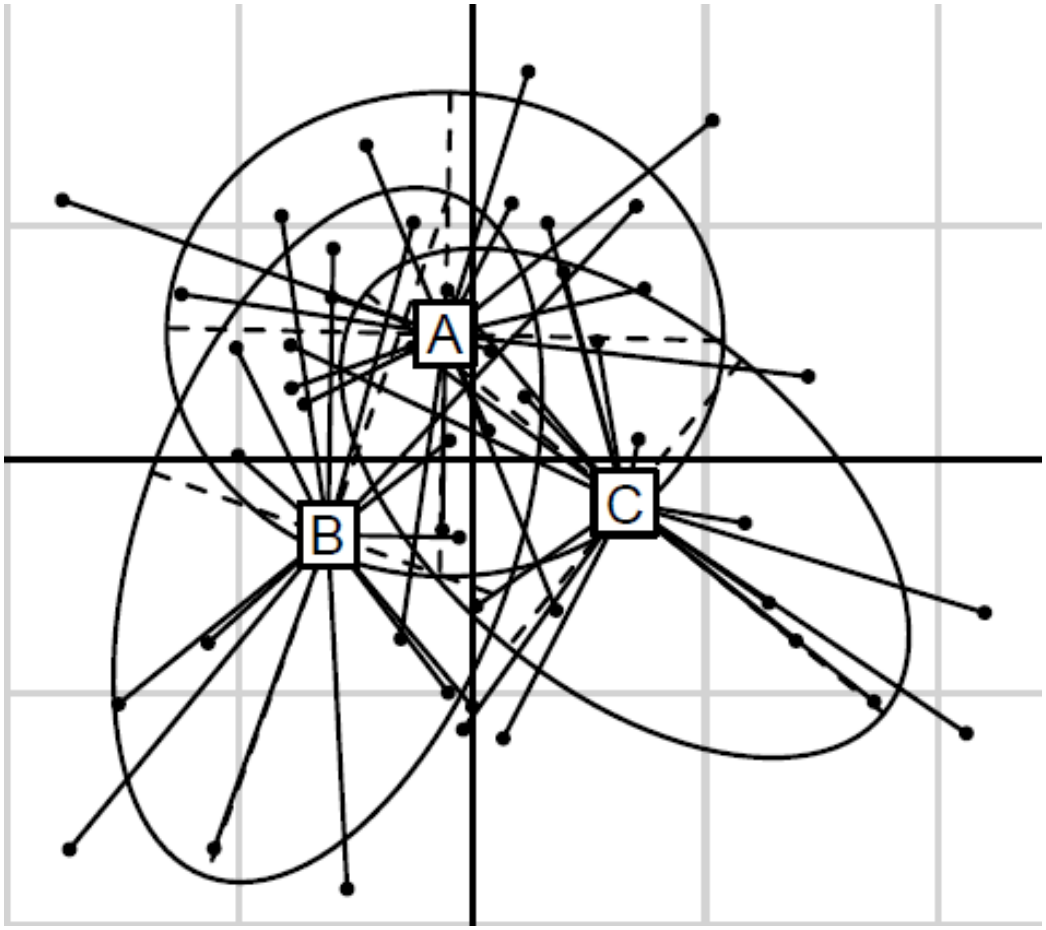

Figure 1.1: Graphical output of the between class analysis (BCA); A = R0, B = R33 and C = R66; Data indicates a significant difference between R33 and R66 ($p<0.05$).

between the treatments (Fig. 1.1, observation = 0.07), especially between the dietary groups R33 and R66. The significance of this analysis was confirmed with a permutation test (P = 0.0447, 10 000 permutations).

Gene expression analysis

A significant effect of diet on the overall relative expression patterns of the investigated genes was observed (MANOVA, F = 1.67, P < 0.05 according to Pillai's trace test). Six genes (HSP70, IGF1, Tf, COX, LMP7 and DFAD) were found to be significantly affected by nutrition/rapeseed substitution: In R33, COX was up-regulated and DFAD was down-regulated. In R66, both IGF1 and Tf were down-regulated. Further, while not significantly different to the control, HSP70 and LMP7 were oppositional expressed between the two treatment groups. LMP7 yielded a higher mRNA abundance in R33, while transcription rate of HSP70 was higher in R66. The results for one gene (GAPDH) displayed an almost significant (P < 0.06) tendency towards down-regulation in both treatment groups compared to the control (Fig. 1.2).

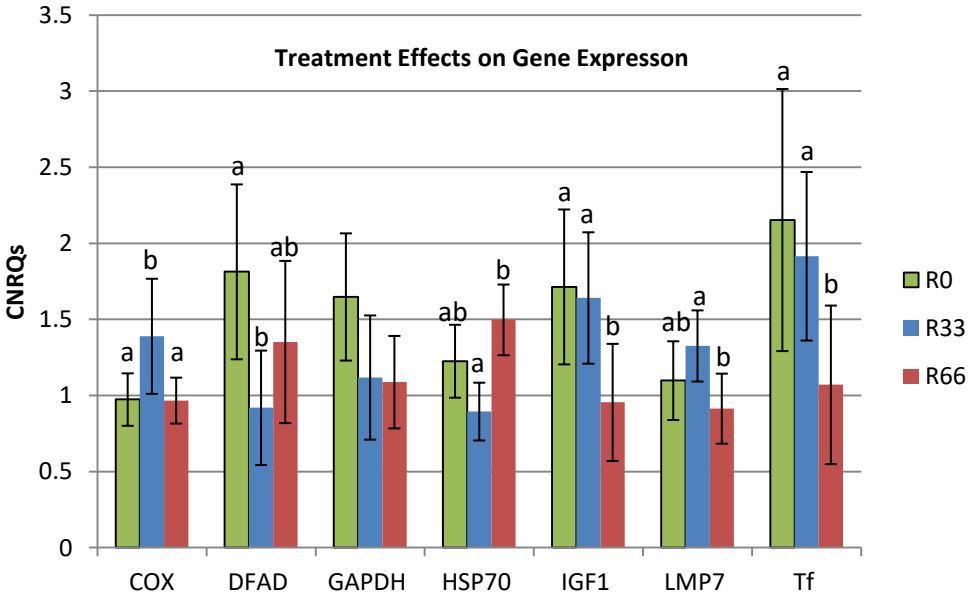

Figure 1.2: Comparisons of the calibrated, normalized relative quantities between the experimental groups. Significantly different groups are indicated by different letters (p<0.05). Only genes affected by the treatment are shown, including the almost significant result for GAPDH. Error bars indicate the standard 95% confidence interval.

Discussion

Divergent gene expression between treatments

We were able to identify alterations in the mRNA abundance of several target genes in turbot liver depending on the amount of rapeseed substitution in turbot aquafeed. These changes predominantly affected genes associated with energy, ion and fatty acid metabolism, as well as stress and immune response although trends with increased RPC inclusion were not always consistent. Hence, data indicated adjustments in the metabolic regulation due to chronic consumption of RPC-substituted fishmeal diets. In addition, the comparison of mean fish condition confirmed a significant difference between R66 and the two other groups. In line with the observations from Slawski et al. (2011), addressing different phenotypes of turbot treated under the same experimental conditions, we are confident to assume that other physiological traits, such as an impaired SGR or a reduced FI, are affected in a similar way (Table 1.3).

We observed that different genes responded in R33 and R66, respectively. Antinutrients, such as GLS, exert their maladaptive effects in two ways: first, by downgrading the palatability of the pellets and therefore reducing feed intake, and second, by impeding access to nutrients and minerals in the diet (Francis et al. 2001). Both factors have been demonstrated to affect thyroid activity in fish (De Pedro et al. 2003, Burel et al. 2000a,b). As thyroid hormones (TH) are known for their crucial role in growth and metabolism (Yen 2001, Hulbert 2000), including regulation of gene expression (Enrıquez et al. 1999, Soboll 1993), a change in their respective concentrations may, at least partially, provide an explanation for the observed pattern.

In turbot, glucosinolate metabolites reduce plasma thyroxin (T4) and triiodothyronine (T3) at concentrations of 3.6 µmol g^{-1} and >4.4 µmol g^{-1}, respectively (Burel et al. 2000b). The authors demonstrated the higher resilience of plasma T3 to dietary glucosinolates to be the result of a compensatory effect, mediated by an increased activity of deiodinase II. Potentially, phytic acid may exert GLS-like effects and, therefore, add to the reduction of TH levels. Furthermore, reduced FI in R66 may reinforce effects of dietary antinutritional factors. Therefore, the divergent pattern of gene expression observed in our treatment groups may reflect actions of chronically

different thyroxin levels. Possibly, turbot in R66 also have reduced T3 levels, as the compensatory deiodinase II effect may be reduced or nullified. However, a contribution of factors other than those considered in this study cannot be excluded, and the ultimate cause remains elusive. Much more research is required to clarify details of the underlying mechanism resulting in uneven transcriptional regulation.

Metabolic adjustments to the experimental diets

The only commonality we observed in both protein replacement diets, R33 and R66, was an almost significant trend towards a decreased transcription rate of GAPDH relative to a purely fishmeal-based diet. This enzyme is an integral part of the glycolysis pathway and therefore relevant for cellular energy metabolism. However, as recent studies discovered various additional functions of GAPDH independent of its involvement in glycolysis (Sirover 2012, 2011, 2005), the exact reason for the detected tenor in GAPDH transcription remains unclear. Due to these additional roles in other biological processes, it is even possible that the GAPDH transcription profile may actually mask further differences between the both treatment groups rather than indicating a common response to dietary antinutrients.

Two genes, exclusively expressed differentially between R33 and R66, are LMP7 and HSP70. The mammalian homologue of LMP7 functions as an immunoproteasome subunit and, therefore, is associated with the catalytic protein metabolism and immune defence. HSP70 is one of the best studied representatives of a super family of molecular chaperones, a large and highly conserved group of proteins, assisting other native proteins in folding into their correct 3D structure, as well as in their transport through the cytosol. This protein has been shown to be associated to stress functions in numerous studies, across multiple taxa (Roberts et al. 2010; Yamashita et al. 2010, Basu et al. 2002). The effect between R33 and R66 appear to be antithetic, with HSP70 being more strongly expressed in R66 and LMP7 being more strongly expressed in R33. However, both genes show no difference in transcript abundance when compared to the control. Hence, a stress or immune response may be excluded as an explanation for this observation. Both, proteasomes and chaperones (cytosolic as well as proteasome-bound analogues) are part of the cellular protein quality control machinery (Bukau et al. 2006). Therefore, results most likely indicate that treatment groups differ in their respective strategies to prevent

the accumulation of miss-folded proteins in the cell, thereby contributing to the overall impression of a dose-dependent adjustment to RPC on the transcriptome level.

On top of the differentially expressed genes between the two treatment groups, juvenile turbot in R33 displayed transcriptional alterations related to cellular respiration and lipid metabolism. Cytochrome c oxidase subunit I was ~1.4-fold up-regulated relative to both the R66 group and the control R0. As the terminal receptor of the respiratory electron transport, the cytochrome c oxidase holds a key role in the energy metabolism. Changes in activity or concentration of this protein are in general considered to indicate shifts in overall metabolic rates of the cell (Nogueira et al. 2001). As a result, fish in R33 may reflect a state of increased ATP requirements.

The second gene transcript exclusively affected in this treatment group was the delta-6 fatty acid desaturase (DFAD), displaying a twofold down-regulation compared to the control group. This enzyme is essential for the synthesis of several LC-PUFAs from 18C PUFA precursors, not only in fish, but a vast range of different taxa (Vagner & Santigosa 2011, Tocher 2003). In general, carnivorous marine fish species, such as turbot, are assumed to have a weak capacity to bio convert C-18 precursors into HUFAs and hence require the preformed LC-PUFA in their diet (Vagner & Santigosa 2011). An increase of mRNA levels relevant for the synthesis of LC-PUFAs has been demonstrated to translate into increased enzymatic activity of this pathway (Zheng et al. 2005, 2004). Therefore, hepatic *de novo* synthesis of several PUFAs may be reduced in this treatment group.

Based on the available data, it is impossible to pinpoint the exact cause for the reported down-regulation of DFAD. Several studies on the impact of experimental diets on liver lipid metabolism in other fish species reported results opposing those presented here. For example, rainbow trout (*Oncorhynchus mykiss*) displayed a higher capacity for lipogenesis after fed a diet containing 100% plant protein material (Panserat et al. 2009) and increased gene expression of enzymes involved in cholesterol biosynthesis and in the fatty acid desaturation in response to a 100% vegetable oil substituted meal (Panserat et al. 2008). Similar results were obtained for Atlantic salmon (Salmo salar), additionally demonstrating the translation of DFAD mRNA levels into tissue lipid composition (Morais et al. 2011). Therefore, contrasting pattern in gene expression may be best explained by species- and dietary-related differences.

IGF1 liver expression decreased significantly in response to the highest tested RPC inclusion level (Fig. 1.2), reflecting reduced feed intake and growth for this treatment (Table 1.3). This enzyme exhibits pleiotropic effects: In addition to its major role in the mediation of growth and development, IGF1 is also involved in the response to salinity stress (Meier et al. 2009, Iwatani et al. 2005), the regulation of apoptosis and immune system activity (Perez-Sanchez 2000). Hence, due its involvement in the response to various insults, IGF1 can be considered a mediator of allostasis (McEwen & Wingfield 2010, 2003). The enzyme's activity is mainly regulated by the growth hormone (GH), which is secreted by the thyroid and binds to specific receptors of the target cell (GHr), which in turn activates (among other enzymes) IGF1 (MacKenzie et al. 1998). Both, fasting and malnutrition, have been demonstrated to reduce the abundance of GHr in hepatic cell membranes, resulting in reduced IGF1 concentrations (MacKenzie et al. 1998; Deane & Woo 2009). In line with our observation, previous studies on turbot demonstrated the concentration of this protein in the plasma to be positively correlated with specific growth rate and food intake (Imsland et al. 2007). Also, in agreement with our data, the authors noted no connection of IGF1 serum levels and the food conversion ratio, which explains why this gene displays no altered expression profile in R33.

The other gene found to be down-regulated in R66 was transferrin, a key enzyme in the iron metabolism with further implications for growth and cellular homoeostasis. It regulates the iron concentration in the serum by binding to iron ions and, subsequently, reacts with the membrane-associated specific transferrin receptor, which triggers the iron uptake via endocytosis (Wang & Pantopoulos 2011). Although it is expressed in several organs, the highest expression levels are in general observed in the liver, because this organ functions as the main storage of iron in most organisms. In fish, maintenance of optimal hepatic iron and blood haematocrit concentration seem to depend on a certain threshold in dietary iron concentration (Pan et al. 2009). Due to the iron-chelating effects of phytic acid, reduced transcription rates may be primarily caused by an iron deficiency and, therefore, imply potential malnutrition.

However, this enzyme has also been recognized as part of the humoral innate immune system, where it comprises several additional functions (Gómez & Balcázar 2007): it impairs pathogen duplication by abstracting the iron, required for bacterial growth, at infection sites and inflamed tissue. In addition, it activates fish

macrophages (Stafford & Belosevic 2003). In humans, transferrin is known to decrease in serum levels during inflammation (Ritchie et al. 1999). However, we did not observe any signs of damaged liver tissue, nor did we observe alterations in the expression of other cytokines (e.g. TNF or IL1), which is why we assume this explanation as less likely.

Implications for Aquaculture and animal welfare

The potential RPC-dependent dose–response, as represented by the divergent transcriptional adjustments between treatment groups, may indicate a hormetic reaction in fish fed the R33 diet. Hormesis describes a process in which low levels of an otherwise harmful stressor elicit a beneficial adaptive response (Mattson 2008). Hormesis has been demonstrated to be an ubiquitous event, occurring across a diverse range of taxa and organizational levels (Calabrese 2008; Calabrese et al. 2013). Involved mechanisms also comprise alterations in gene expression and have been linked to diet composition and energy availability (Mattson 2008). Therefore, fish in this group may even benefit from enhanced robustness to secondary stressors, as the implied increase in metabolic activity may alleviate coping with additional stressors.

On the other hand, treatment R66 reduced feed intake, bioavailability of several nutrients and subsequent energy availability, which may have further implications for fish welfare. Although gene expression analysis did not provide conclusive evidence for a stress response, turbot may be more susceptible to challenges, as they may fail to fuel a sufficient allostatic response (McEwen & Wingfield 2010, 2003). Further, Korte et al. (2007) highlighted the importance of an organism's ability to respond adequately to a stressor, as well as the efficient termination of this reaction after the challenge has passed. This requires the organism to keep control over the concentrations of all involved enzymes. As described above, down-regulation of receptor-controlled IGF1 and Tf may hint towards a change in the abundance of respective receptor-proteins in the membrane. Hence, regulatory capacity of fish fed the highest tested RPC substitution level may be impaired. Due to its central role for downstream signal cascades, impeded control over IGF1activity may hinder switching between metabolic pathways. In addition, as fish challenged with pathogens tend to increase transferrin production (e.g. Ercan et al. 2013, Chettri

et al. 2012, Liu et al. 2010,), fish under the R66 nutrition may be more prone to bacterial infection, as they may not be able to elevate serum transferrin to a suitable level in response to a contagion.

In conclusion, complementing the physiological data published by Slawski et al. (2011), our results underline the assumption of a safe inclusion of up to 33% RPC in the diet of juvenile turbot. Moreover, minor concentrations of antinutrients may induce a hormetic reaction, which than could foster the turbot's robustness to additional challenges. RPC inclusion levels of 66%, however, not only appear to be unfavourable with respect to important production parameters but may also impair fish welfare by reducing the ability to counter challenges as indicated by our data. Most likely, these outcomes are not exclusively evoked by direct antinutritional effects, but also by reduced feed intake. Hence, an improvement of palatability may increase the turbot's acceptance of higher RPC substitution rates and reduce potential adverse consequences. Both IGF1 and Tf may have the potential to serve as suitable biomarkers to evaluate the nutritional status of juvenile turbot, as well as a contingent increased susceptibility to stress. However, more research effort is required to test and reassure these assumptions beyond the stage of first exploration. Despite the investigation of gene expression in multiple genes correlated with metabolism, stress and immune defence, the picture provided by our study only represents a small glimpse and further studies, applying broad range methods like RNA sequencing, are required to provide a more comprehensive picture.

Acknowledgements

This study was financially supported by the German Federal State of Schleswig-Holstein and the European Regional Development Fund (ERDF) through the NEMO project (122 08 005). The authors gratefully thank Dr. Hanno Slawski for his comprehensive contribution to this paper. We further thank Katrin Beining for her kind support in the lab, including primer assay development. The staff of the Genetic Diversity Centre in Zurich, especially Tania Torossi, is acknowledged for technical assistance during gene expression analysis.

CHAPTER II

Divergent gene expression in the gills of juvenile turbot (*Psetta maxima*) exposed to chronic severe hypercapnia indicates dose-dependent increase in intracellular oxidative stress and hypoxia

Abstract

Elevated concentrations of carbon dioxide are a common stressor for fish and other aquatic animals. In particular, intensive aquaculture can impose prolonged periods of severe environmental hypercapnia, manifold exceeding CO_2 concentrations of natural habitats. To cope with this stressor, gills are essential and constitute the primary organ in the acclimatization process. Yet, despite a general understanding of changes in ion regulation, not much is known regarding other cellular mechanisms. In this study, we apply RT-qPCR to investigate changes in the expression of several genes associated with metabolism, stress and immunity within gills of juvenile turbot (*Psetta maxima*) after an eight-week exposure to different concentrations of CO_2 (low = ~3000 µatm, medium = ~15000 µatm and high = ~25000 µatm CO_2). While additional histological examination of the gill tissue only found a significant increase of hypertrophied secondary lamella in the highest tested treatment level, gene expression results implied both, common and dose-dependent transcriptional adjustments. Common up-regulation of IL-1ß, LMP7 and Grim19 at medium and high hypercapnia indicated an increase of reactive molecule species (RMS) within gill cells. Contemporaneous increase in Akirin and PRDX transcripts at medium CO_2 indicated enhanced anti-oxidant activity and regulation of transcription, while reduced mRNA concentrations of COX, EF1α and STAT2 at high CO_2 denoted suppressed protein synthesis and reduced metabolic capacity. In addition to up-regulated DFAD and ApoE expression, implying compensating repair measures, gills exposed to the highest tested treatment level seemed to operate close to or even beyond their maximum capacity. Thus, fitting the model of capacity limitation, our results provide evidence for accretive intracellular hypoxia and oxidative stress in the gills of turbot, dependent on the level of environmental hypercapnia. Further, genes, such as COX, may be

valuable biomarkers when attempting to discriminate between a successful and an overpowered stress response.

Introduction

Environmental hypercapnia, the noteworthy increase in carbon dioxide concentrations in an organism's surrounding, can act as a potent stressor for many aquatic life forms. The topic has garnered much attention in the light of man-made increase in atmospheric CO_2 levels and the resulting future acidification of the oceans. This event is expected to challenge a vast range of species, especially on the most vulnerable early life history stages. In addition, environmental hypercapnia has also become of great relevance in the realm of intensive aquaculture. In particular with regard to land-based recirculating aquaculture systems (RAS), microbial respiration and high rearing densities can elicit periods of severe hypercapnia (Kristensen et al., 2009, Colt, 2006). Occasionally, these factors are pronounced or occur in concert, which can lead to a prolonged, severe derailing of dissolved pCO_2 from a species dependent optimum.

Due to the ability of carbon dioxide to quickly diffuse across cell membranes, physiological pCO_2 values in fish usually increase parallel to those in the environment (Moran and Støttrup, 2011, Fabry et al. 2008, Pörtner et al. 2004, Seibel & Walsh 2003). Within the organism, hypercapnia causes an overall increase in proton concentrations, thus, posing a threat to intra- and extracellular pH equilibria (Fivelstadt 2012, Ishimatsu et al. 2004, Pörtner et al. 2004).

Usually, fish are able to counter disturbances of the acid-base balance by accumulating bicarbonate in the body fluids and reducing proton concentrations via energy costly ion exchange processes (Fabry et al. 2008, Ishimatsu et al. 2004, Pörtner et al. 2004, Seibel & Walsh 2003). As the buffering capacity of gill breathers via bicarbonate is limited, ion regulation constitutes the primary coping mechanism, in particular under conditions of severe environmental hypercapnia (Evans et al. 2005, Pörtner et al. 2004). Thus, the gills play an essential role in the acclimatization process, as this organ can account for up to 90% of total ion regulation capacity (Claiborne et al. 2002). Indeed, despite a potential reduction of performance traits - such as the specific growth rate - fish survive pCO_2 concentrations which are many times higher than those in their respective natural habitats once the gills are fully

developed (e.g. *Sparus aurata*, Ben-Asher et al. 2013; *Gadus morhua*, Moran & Støttrup 2011; *Dicentrarchus labrax*, Petochi et al. 2011; *Anarhichas minor*, Foss et al. 2003 *Salmo salar*, Fivelstadt et al. 1998).

Yet, as a vital element of the stress response, environmental hypercapnia can challenge the gills. However, histopathological changes of the gill tissue are often of limited specificity, as various environmental stressors appear to elicit comparable symptoms (Harper & Wolf 2009). Thus, it can often be difficult to distinguish between an adaptive response, triggered in order to restore homeostasis, and overcome pathologies, evoked by an already disrupted metabolism. The application of molecular methods, however, offers deeper insights in the metabolic response of hypercapnia and the associated acidification of the water.

While poorly understood in its entirety, elaborate scientific effort has identified the main processes involved in ion regulation in the gills during normcapnic and hypercapnic conditions (Evans et al. 2005, Claiborne et al. 2002), including changes in the expression of the respective genes during acute and chronic exposure (Deigweiher et al. 2008). While there is evidence that the response to hypercapnia comprises extensive changes in transcription and protein synthesis beyond the scope of ion regulation (Deigweiher et al. 2010), not much is known with regard to other molecular adjustments. In a recent study, following the exposure to ~15,700 µatm carbon dioxide for 1 h, Dennis et al. (2015) found an increase in c-fos mRNA in the gills of bluegill (*Lepomis macrochirus*), while silver carp (*Hypophthalmichthys molitrix*) increased hsp70 and hsc70-2 transcript abundances. This identifies species dependent differences in the coping strategy during the acute phase. Proteome studies in the gills of the Atlantic halibut (*Hippoglossus hippoglossus*) suggested concentrations predicted for future ocean acidification scenarios (1000 µatm pCO_2) to increase the abundance of enzymes related to energy metabolism, cellular turnover and apoptosis, hence, implying profound biochemical adjustments in the long run (Bresolin de Souza et al. 2014).

In this study, we aimed at further illuminating the underlying mechanisms involved in the cellular adjustment to chronic environmental hypercapnia, especially when extreme carbon dioxide concentrations may force the gills to operate close to, or even beyond, their maximum ion regulation capacity. After an eight-week exposure to different regimes of severe hypercapnia (low = ~3000 µatm, medium = ~15000 µatm and high = ~25000 µatm CO_2), we examined the expression of 40 different

genes in the gills of juvenile turbot (*Psetta maxima*). By focusing on transcripts related to metabolism, cellular homeostasis and the immune defence, we also hoped to infer reliable information regarding the gills condition, including their ability to resist potential additional stressors. Further, we included classical stress indicators, such as HSPs, to test their soundness as biomarkers under conditions of chronic stress. The additional incorporation of histological data allowed us to compare the effects of the respective treatment level on two different scales of biological organization. Thereby, investigating sever levels of carbon dioxide within an enclosed RAS not only provides an interesting system to study chronic stress, but also serves as an extreme model for ocean acidification.

Methods

Experimental Setup

The experiment was conducted in a recirculating aquaculture respirometer system (RARS) (an in-depth description is provided by Stiller et al. 2015 and 2013), comprising 10 tanks with a volume of 250 l each, as well as a set of different water chemistry analyzers recording oxygen (O_2) concentration (amperometric electrode, dTRANS O2 01; JUMO GmbH & Co. KG, Fulda, Germany); water pH (pHNBS, intermediate junction electrode, Ionode IJ44, TPS Pty Ltd, Brisbane, Australia); temperature; total ammonia nitrogen (TAN, loop flow orthophtalaldehyde fluorometric autoanalyzer, µMac, SYSTEA S.p.A., Anagni, Italy) and dissolved carbon dioxide (purpose built flow through headspace analyzer, MK-2 pCO_2/Fast Analyzer (SubCtech GmbH, Kiel, Germany).

Juvenile turbot used in this study were procured from the Maximus A/S fish farm (Bedsted, Denmark) and acclimatized for two months prior to the onset of the trials. Each tank but one was studded with 14 fish (mean weight ~55 g). The animals were fed ad-libitum once per day with a commercial diet (ALLER 505 EX 9 mm, ALLER Aqua, Christiansfeld, Denmark, macro nutrient profile: crude protein 50%, crude fat 16%, ash 9%, fiber 1%).

In order to simulate different degrees of sever hypercapnia, tanks were divided into three groups with three replicates each. The tenth tank was left empty and used as a reference. CO_2 was infused via fine pore ceramic diffusers (model

MBD075, Point Four Systems Inc., Canada) into six tanks. The desired apportionments were achieved with an adjustable needle valve and low volume flow meter.

In three tanks, carbon dioxide concentrations increased due to passive diffusion effects in the RARS, and stabilized at the lowest treatment level achievable for the system. Medium and high CO_2 treatment levels were achieved by gradually ingassing over the time period of seven days, which allowed the system to equilibrate and the turbot to acclimate to the new conditions. Final CO_2 concentrations for the span of the experiment were labelled as low (~3000 µatm), medium (~15000 µatm), and high (~25000 µatm).

Measurements were sequentially conducted multiple times a day (9-18 day^{-1}, dependent on the cumulative time it took the different analyzers to complete a measurement cycle). Ammonia concentrations were determined weekly. In addition, nitrite was measured via a commercial kit, while salinity was assessed using a refractometer, both on a daily basis. Weekly measurements of alkalinity were conducted with a potentiometric titration using an acid standard.

At day fifty-six of the experiment, gills were dissected from five individuals per tank and a fraction was put into RNAlater (Qiagen, Hilden, Germany) for further gene expression analysis. The remainder was fixed in 4% phosphate-buffered formalin in preparation for histological examination. Therefore, gill samples were subsequently dehydrated in a graded series of ethanol and embedded in paraffin. Samples were sectioned at 5 µm with a rotary microtome and stained with hematoxylin-eosin (HE). Finally, information regarding hyperplasia, hypertrophy and lamellar clubbing was retrieved via optical analysis, applying a microscope equipped with a digital camera. Data related to the overall growth rates, condition indices, haemoglobin or protein turnover, along with additional details about the experiment can be found in Stiller et al. (2015).

RNA extraction, quality assessment and cDNA synthesis

20 mg of tissue per gill sample were subjected to total RNA extraction via the Invitrap® Spin Tissue RNA Mini Kit (Stratec, Berlin, Germany) following the manufacturers' instructions. RNA quality was assessed using the Experion® System (Biorad, Munich, Germany), in which a RQI of 8.0 or higher was set as the standard for further downstream applications. The QuBit® 2.0 Fluorometer (Life technologies,

Carlsbad, California) was used to determine the respective RNA quantities of the samples. 800 ng of total RNA per sample were deployed for the next working step. The synthesis of cDNA was conducted via the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), which incorporates the digestion of genomic DNA before reverse transcription. For every sample, an aliquot of gDNA digested RNA was taken and used as a -RT control during gene expression analysis. The manufacturers guidelines for reverse transcription were modified to improve cDNA synthesis: The gDNA wipe-out step was elongated to five minutes and reverse transcription was conducted for twenty minutes instead of fifteen.

Primer design and gene expression analysis

A total of 40 genes of interest (GOIs) were analysed in this study. In order to make assumptions regarding the cell's condition, as well as to identify mechanisms relevant for coping under severe chronic hypercapnia, GOIs were chosen to represent a vast range of cellular functions. Therefore, the set of primers developed in Hermann et al. (2016) was used and extended to include additional genes relevant for stress and immune responses (Table 2.1). GenBank (http://www.ncbi.nlm.nih.gov) was used to obtain sequence information of the additional GOIs on the basis of which primers were designed via PRIMER3 (Untergasser et al. 2012, Koressaar & Remm 2007). PCR was conducted on the BioMark® HD System (Fluidigm, San Francisco, USA), applying 96 x 96 Dynamic Array gene expression chips. To evaluate primer efficiencies, each chip comprised three different cDNA dilution series ranging from a 1:2 to a 1:6250 ratio. Further, each chip was charged with three non-template controls and three RT-controls.

Table 2.2: Results of the evaluation of reference genes by the NormFinder Algorithm.

Reference Gene	M Value	CV
CSD	0.281	0.109
GAPDH	0.292	0.116
Tubα	0.289	0.114
average	**0.287**	**0.114**

Table 2.1: PCR primer sequences, accession numbers and calculated efficiency.

Gene	Abbr.	Accsession No	Primer Sequence	Eff%
HMG-CoA-reductase	HMG-CoAr	JN542428.1	fw: TGCTGGAGTTGTCCCGTGAGAGT	108.62
			rev: CTGTGGGCGTGAACCATCACCAA	
Adenylat Kinase 2	ak2	DQ848978.1	fw: ACGACTTGCTGGACAAGAGACGC	100.8
			rev: GCGGCCACTGGGCTGATGAATTA	
Akirin	Akirin	HM237345.1	fw: GACCATGAGGAGAAGATCCGGGAGG	107.5
			rev: TCAGGAGACATAACTAGCAGGCCGG	
Alpha tubulin	TubA	DQ848853.1	fw: GCCCTACCCTCGTATCCACT	100.1
			rev: TGATGTCAGCCACAGAGAGC	
Apolipoprotein E	apoE	AJ236883.1	fw: AGGCCACCGCTAAGGAGCTTTTCA	102.53
			rev: TTCCCAACCTGCTCTTGGATCTGGG	
Arginase Type II	Arginase	AF467774.1	fw: ACGCGGACATAAACACGCCCA	99.82
			rev: TGGCTGGCATCTTGTCTTGGAGC	
Cathepsin D	CathD	EU077233.1	fw: TCGCCTGCTTGCTTCACCACA	107.93
			rev: GCCCGACAAACTGCCAGATCCAT	
Cell death-regulatory protein	GRIM19	FJ617007.1	fw: TCCAGGTTGGAAGGTCGGTGAGA	105.34
			rev: CGTACCACAAGAAGCCAAAACGCT	
Cold-shock domain	CSD	DQ840135.1	fw: ACGGCGAGGAGGACAAGGAGAAT	103.56
			rev: TTGAAGTTGCGGCGGTAGCGA	
Copper chaperone	CSS	DQ400684.1	fw: AACCCGCAGTTTTCGACACGTCA	104.9
			rev: TGAACAGCCCTCGCACGTCATC	
Cystatin B	CystB	HM237342.1	fw: CATGAAGCCCCACGCAGAGGAAAA	95
			rev: AGTAGTTGGTGCCGCTCACAAGC	
Cytochrome P4501A	Cyt450	AJ310694.1	fw: ATCGCTCTCCTCTTCTCTCT	99.41
			rev: TTAGAGGTGCAGTGTGGAAT	
Cytochrome-C-oxidase subunit 1	COX	DQ848855.1	fw: TCGCGCTTACTTCACCTCCGCTA	99.92
			rev: CATGGAGTGTTGCGAGCCAACTGA	
Elongation factor 1 alpha	EF1α	AF467776.1	fw: ACCGAGGTGAAGTCTGTGGA	102.3
			rev: CGGACACGTTCTTGATGTTG	
Glutathione S-transferase	GST	DQ848966.1	fw: GGGTTCGCATCGCTTTT	89.88
			rev: GGCCTGGTCTCGTCTATGTACT	
Glycerinaldehyde-3-phosphate dehydrogenase	GAPDH	DQ848904.1	fw: CAGTGTATGAAGCCAGCAGAG	111.31
			rev: ACCCTGGATGTGAGAGGAG	
Growth arrest and DNA-damage inducible protein 45 alpha	Gadd45	EU711049.1	fw: AGGACGTGGCCCTCCAAATCCAT	108.9
			rev: TGCCAGACGCCTCATGTTGCT	
Growth hormone receptor	GHr	AF352396.1	fw: CCCTCATCCAAACCCAAAC	95
			rev: GGGCATAACATTGCTGACCT	
Heat shock protein 40kDa	HSP40	BT026744.1	fw: AAGAAAGGTGCCCTGGAGAAGTGCT	94
			rev: TGGCAGTCGGAACACATGCTTTGG	
Heat shock protein 70kDa	HSP70	EF191027.1	fw: CCGCTGCTGCTATTGCCTATGGT	100.46
			rev: TGCCGCCACCGAGATCAAAGATG	

Table 2.1 (Continued)

Heat shock protein 90kDa	HSP90	EU099575.1	fw: ATCAACAACCTGGGAACCATCGCC	103.25
			rev: GAAACCCACGCCAAACTGACCGA	
Insulin-like growth factor 1	IGF1	FJ160587.1	fw: TGTACTGTGCGCCTGCCAAGACTA	102.55
			rev: TGCTGTGCTGTCCTACGCTCTGT	
Insulin-like growth factor 2	IGF2	JN032705.1	fw: GAATGTTGTTTCCGTAGCTG	90
			rev: TCGGGACTTCCTGTTTTAGT	
Interferone regulatory factor 3	IRF3	JF759908.1	fw: TGCTCGCTGTTCCTCTGCCTT	96.4
			rev: TGAAGTAAGCACCACCTCCACTGTG	
Interferone regulatory factor 5	IRF5	JF913460.1	fw: TCCGAACCCTCCGCCTTTTGAGA	99.6
			rev: TACGGGAACCACCTGGACGATGA	
Interferone regulatory factor 7	IRF7	HQ424129.1	fw: TCATCACAGTCAAGGTGGTC	92
			rev: AGATCGTAGAGGCTGTTGTG	
Interleukin-1 beta	IL1	AJ295836.2	fw: ACCAGACCTTCAGCATCCAGCGT	96
			rev: TTCAGTGCCCCATTCCACCTTCCA	
Lipoprotein lipase	LPL	JQ690822.1	fw: TCCCTTTGTTATGCCTGTCC	93.36
			rev: GCTGATGATTGAGTCCTTCTCC	
Low molecular weight polypeptide	LMP7	FJ617008.1	fw: GGGCATAGTTCACGCTACGCACA	89.85
			rev: CGTGTGTCGGGGTCAAACTCAGAA	
Lysocyme C	LysC	EU747734.1	fw: GAACGCTGTGAATTGGCCCGACT	86.63
			rev: GTTGGTGGCTCTGGTGTTGTAGCTC	
Natural resistance-associated macrophage protein	NRAMP	EU747732.1	fw: CGTCTTTGTGGTGGCTGTCTTTGCT	103.99
			rev: TCCCGGTTGCGTTGCATTGCT	
Peroxiredoxin 1	PRDX	EU747733.1	fw: AGCACACTGACAAACACGGCGA	92
			rev: TGCGGCAGAACATCAAGGAGACC	
Polyunsaturated fatty acid elongase	ELO	AF465520.2	fw: CGTTCCTCCACATTTACCACCACGC	105.01
			rev: TTGAGGGATGCGCCGAAGTACGA	
Protein phosphatase 1 catalytic subunit beta	PP1bc	DQ364569.2	fw: GCGTGGACGAAACTCTGATGTGCT	97
			rev: ATCGCTTCTTTGGAGCTTGGGCTG	
Signal transductor and activator of transcription 2	STAT2	FJ719015.1	fw: CGGCAGCAGAAAACCTGCATTGG	106.77
			rev: TCCCGCACCTGGAACAAACACAC	
Toll-like receptor 3	Tlr3	FJ009111.1	fw: GACGTGCTGATCCTGGTCTTTCTGG	106
			rev: AGCTCAGGTAGGTCCGCTTGTTCA	
Transferrin	Tf	AJ277079.1	fw: ATCGTTGGAGGAGGCGGTAAGCA	99.34
			rev: TGGGTCTTGGAGCAGTCCCCTTT	
Transferrin receptor	Tfr	DQ400685.1	fw: GTTTCTCCCAAAGAGAGTCC	97.1
			rev: GAGGAAATCGAGGTGGTT	
Tumor necrosis factor alpha	TNF	FJ654645.1	fw: AAAAGAAGTCGGCTACGGGGTGGA	93
			rev: TTCCAGTGCCAAGCAAAGAGCAGG	
Δ6-Fatty acid Desaturase	DFAD	AY546094.1	fw: TGGAGAGTCACTGGTTTGTGTGGGT	91.5
			rev: AGGTGGCCTGTAGCTGCATGGTTA	

Data processing and normalization

Software provided with the BioMark® HD System was used to control the quality of the PCR products via melt curve analysis (Fluidigm, San Francisco, USA). All following steps, from further data processing to the calculation of calibrated normalized relative quantities (CNRQs) were conducted with the QBASE software (Biogazelle, Zwijnaarde, Belgium). First, raw data was screened for outliers and extreme values. Triplicates with a standard deviation above 0.5 were omitted from further analysis. Next, the most suitable reference genes were determined with the help of the GeNorm algorithm (Vandesompele et al. 2002). CSD, GAPDH and Tubα were found as the most stable combination (Table 2.2) and their mean was used to calculate normalized relative quantities (NRQs) as follows (Hellemanns et al. 2007):

$$NRQ = \frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[f]{\prod_0^f E_{ref_0}^{\Delta Ct,ref_0}}}$$

Required primer efficiencies were derived from the dilution series included in the chip runs. For each gene, the average crossing point (Cp) across all samples was used as a calibrator. Replicates of each sample x primer combination were conducted on the same chip, so that no inter-run variation had to be taken into account and NRQs could be used for the statistical analysis.

Statistical analyses

Statistics were conducted using the freely available software R (R Core Team, 2016). Both, histological gill data and NRQ values derived from gene expression analysis, were monitored with regard to distribution and extreme values by visual (box-plots, histograms) and mathematical (Shapiro-Wilk normality test) means. To allow for subsequent parametric tests, data was subjected to a box-cox transformation when required.

An ANOVA was used to test for treatment level specific differences in the abundance of gill pathologies. Gene expression data was subjected to a BCA (between class analysis) to check for overall differences in transcription between gills exposed to different carbon dioxide concentrations. NRQs were also analysed with a

multivariate analysis of variance (MANOVA) to confirm possible results of the BCA. Afterwards, affected genes were identified with the help of multiple ANOVAs, followed by Tukey´s HSD post-hoc test in case of significance.

Results

Gill histology

Within the gills dissected from fish exposed to 25000 µatm CO_2, we noticed a significant increase in the percentage of secondary lamella affected by hypertrophy in comparison to the other two groups. Data concerning other histological traits revealed no significant differences between groups (Fig. 2.1).

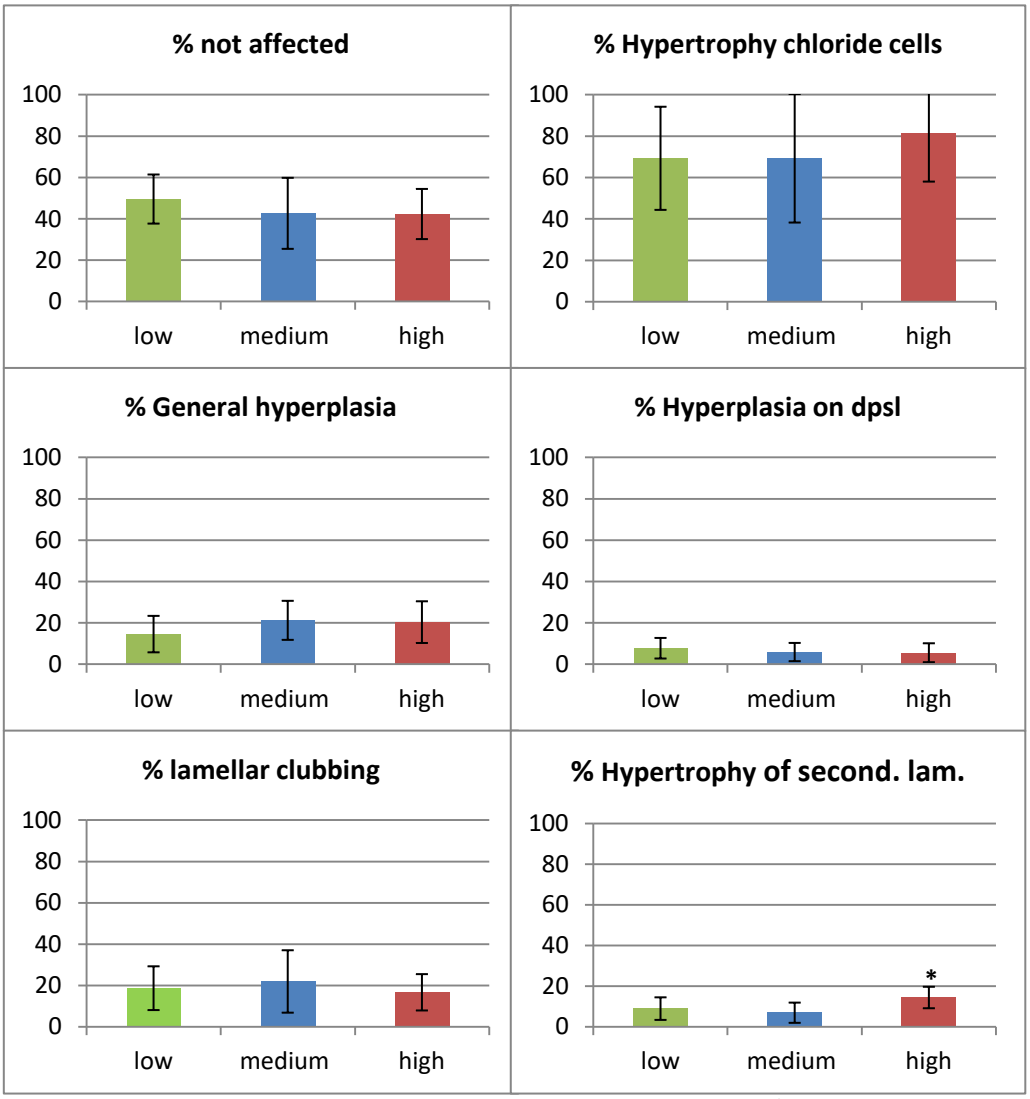

Figure 2.1: Histological analysis of the gill tissue. Error bars are +/-1 standard deviation. Significant different groups are marked with an asterisk (*). Dpsl = distal part of secondary lamellae.

Overall group comparison

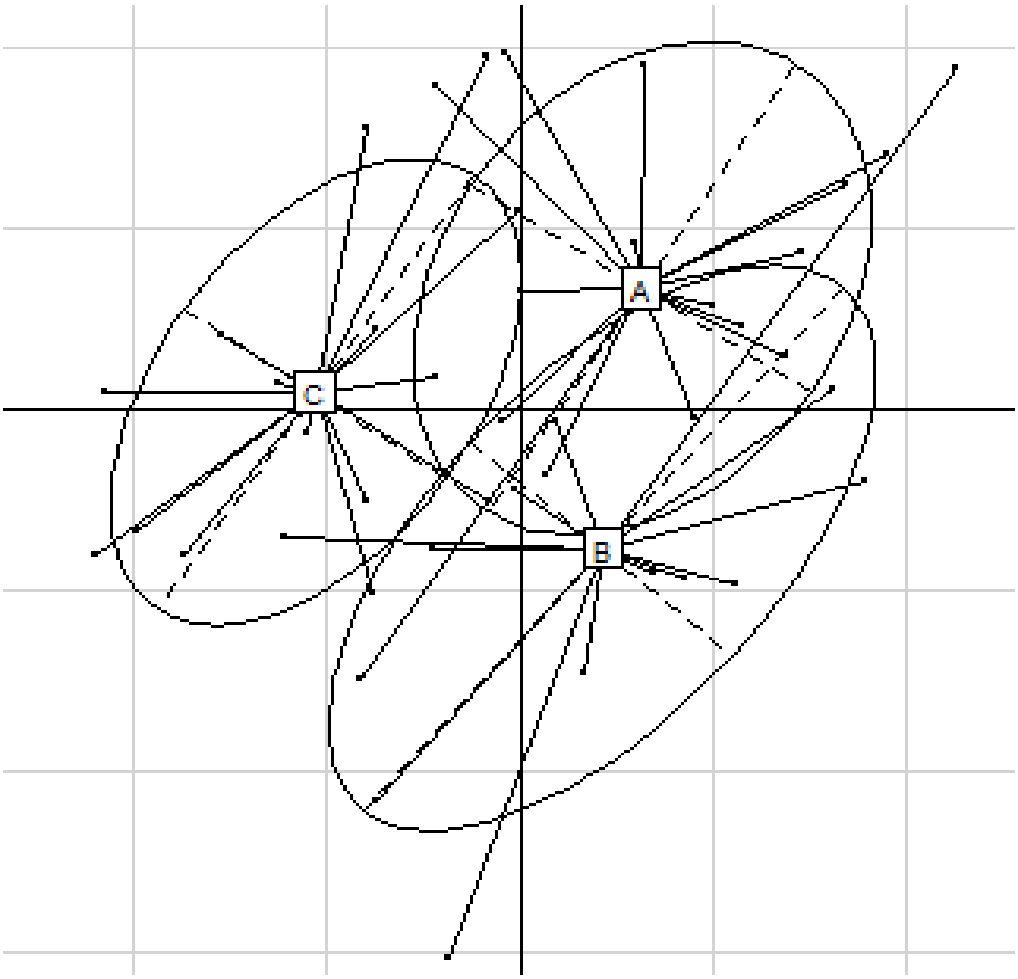

Figure 2.2: Graphical output of the between class analysis (BCA); A = Low, B = Medium and C = High. Data indicates a significant difference between treatment level ($p<0.001$).

Data mining revealed an uneven, bimodal distribution pattern for the gene PPbc1 while no working transformation factor was found. Due to the additional accumulation of various outliers and missing values, the gene was excluded from further analyses in its entirety. Due to technical difficulties one sample assigned to the medium treatment had missing values for the genes CystB, GHr, HSP40, IGF2, IRF3, PRDX, IRF7 and Tfr. The missing values were replaced with the mean values of the respective replicate group.

To determine the suitability of each gene for a factor analysis, the Kaiser–Meyer–Olkin value (KMO) was calculated before the actual BCA. The KMO values of nine genes (TLr3, Tf, ak2, Tfr, HMG, Cyt450, IRF7, IRF5, LysC) were below the recommended value of 0.5, leading to their exclusion from the cluster analysis. The remaining 27 genes yielded an overall KMO of 0.72. In addition, Bartlett's test of

sphericity ensured that relative expression values of deployed genes were not unrelated (chi-square = 784.8, $p < 0.0001$) and therefore eligible for the BCA. The between class analysis found an overall dissimilarity between the three tested groups (Fig. 2.2, observation = 0.135) which was confirmed by a permutation test ($p < 0.001$, 10 000 permutations). Pertaining to the entirety of tested genes, an overall effect of carbon dioxide concentration on transcription was further supported by the MANOVA ($F = 6.1285$, $p<0.01$ according to Roy´s trace test).

Gene expression analysis

In comparison to the lowest tested concentration, IL-1ß, LMP7 and Grim19 were equally up-regulated in both groups, the ~15000 µatm and the ~25000 µatm treatment (Fig. 2.3). Another group of genes responded specifically to one of these two treatment levels (Fig. 2.4 - 2.6): In the medium treatment PRDX and Akirin displayed, additionally, a significant increase in mRNA. In the high treatment, DFAD and ApoE were up-regulated, while COX, EF1α and STAT2 showed a decrease in transcript abundances.

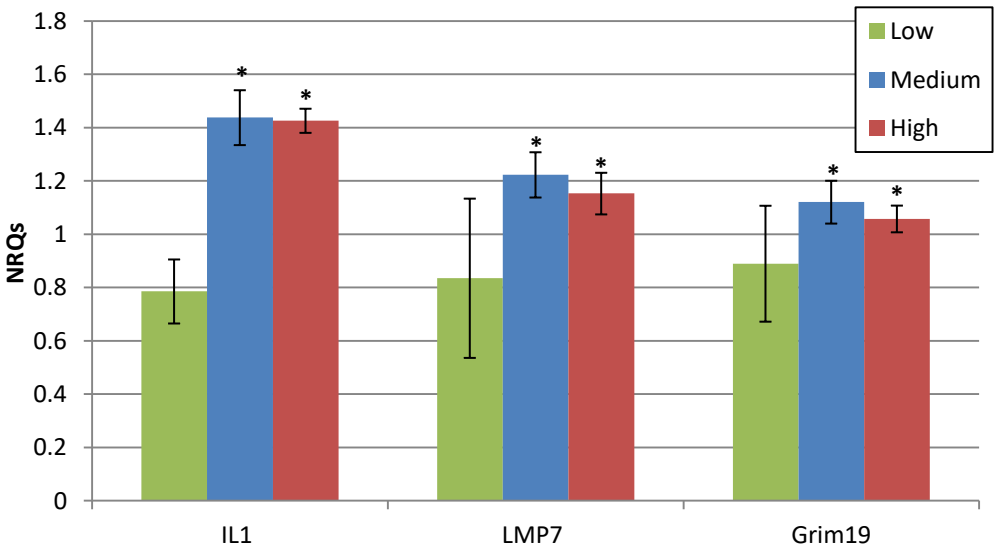

Figure 2.3: Genes affected by both, the medium and the high treatment. Error bars reflect the standard error. Significantly up-regulated groups are marked with an asterisk (*). Low = ~ 3000 µatm CO_2, Medium = ~ 15000 µatm CO_2,High = ~ 25000 µatm CO_2.

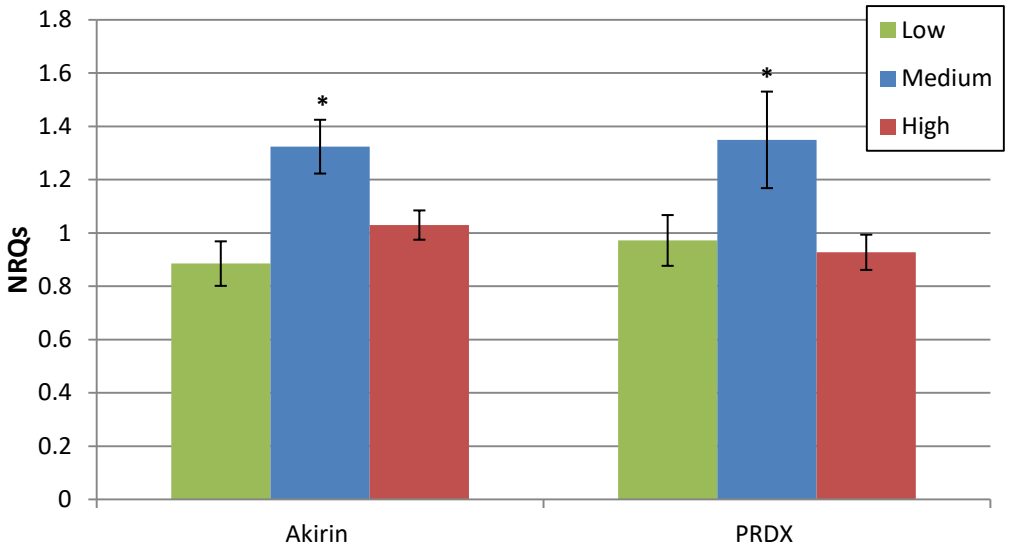

Figure 2.4: Genes up-regulated in the medium treatment. Error bars reflect the standard error. Significantly up-regulated groups are marked with an asterisk (*). Low = ~3000 µatm CO_2, Medium = ~15000 µatm CO_2,High = ~25000 µatm CO_2.

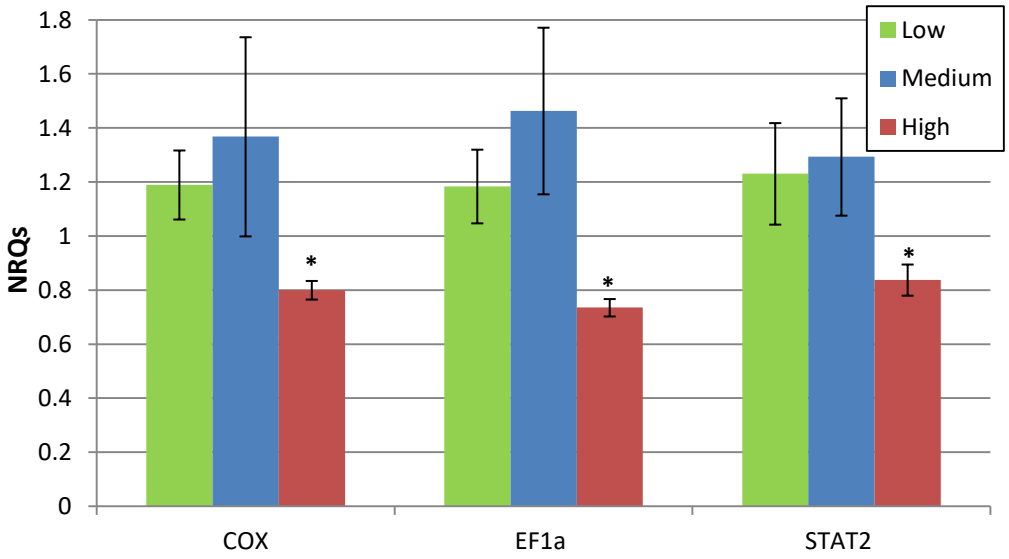

Figure 2.5: Genes down-regulated in the high treatment. Error bars reflect the standard error. Significantly down-regulated groups are marked with an asterisk (*). Low = ~3000 µatm CO_2, Medium = ~15000 µatm CO_2, High = ~25000 µatm CO_2.

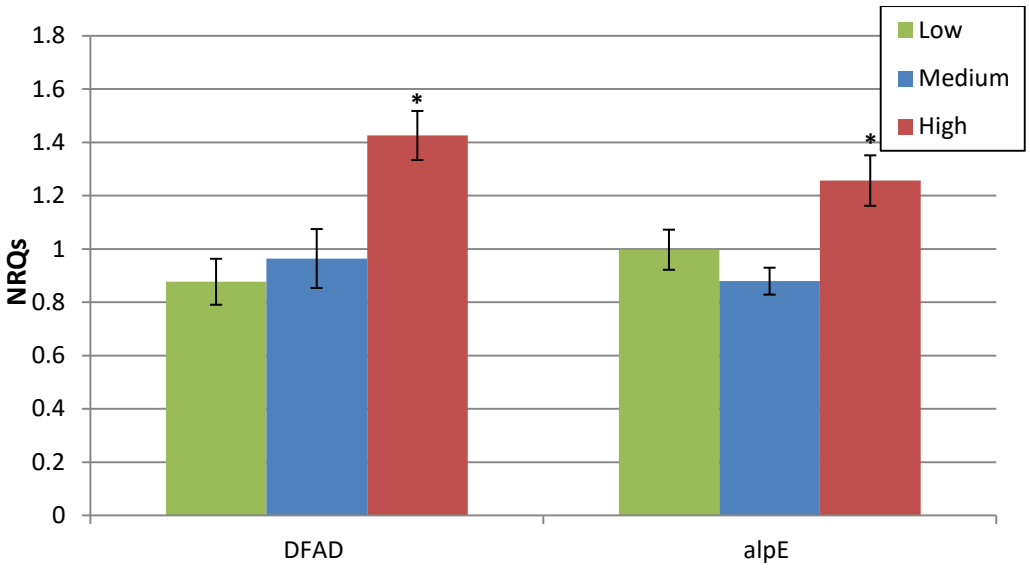

Figure 2.6: Genes up-regulated in the high treatment. Error bars reflect the standard error. Significantly up-regulated groups are marked with an asterisk (*). Low = ~3000 µatm CO_2, Medium = ~15000 µatm CO_2,High = ~25000 µatm CO_2.

Discussion

Hypercapnia induced changes in gene expression

The carbon dioxide concentrations applied in this study greatly exceed those occurring in the natural environment of turbot. Consequently, the constant threat to oxygen tension and acid-base balance allow to assume that animals in all treatment levels experienced various degrees of chronic stress. However, most of the physiological irritations observed in this study appeared to occur independent of the applied treatment level. Only an increase in hypertrophied secondary lamella could be linked to the highest tested concentration of carbon dioxide. As an increased retention of CO_2 within the animal would have to be tolerated, this structural change could be regarded as a trade-off, in which attenuation of environmental hypercapnia outweighs negative side effects. This response may serve as a defence mechanism, aiming for the increase of the diffusion distance of carbon dioxide across the gills (Mallatt, 1985). A comparable reaction was observed in the gills of the walking catfish *Clarias batrachus*, exposed to acidified water (pH = 5, Naskar et al. 2009). However, as a protective measure, hypertrophy usually occurs in concert with other gill reorganizations, as for example demonstrated in turbot gills exposed to elevated

ozone concentrations (Reiser et al. 2011). It is therefore also possible that the observed response reflects the onset of gill damage rather than an actual adaptation to hypercapnia. Yet, all three groups displayed high rates of unaffected secondary lamella with only low abundances of clubbing and hyperplasia. Thus, we considered the gills in general to be equally functional and capable of maintaining their respiratory and ion regulatory functions.

In contrast to ambiguous results derived from the investigation of histological traits, several genes differed in their respective mRNA abundance, indicating a coping response to environmental hypercapnia on the sub-cellular level (Fig. 2.3 - 2.6). Within the obtained expression data, affected genes could be further divided into two sub-groups: those who were similarly up-regulated in both, the medium and the high treatment, suggesting a general response to elevated levels of CO_2 above a certain threshold, and those who changed their expression exclusively in one of the two groups, indicating an additional dose-dependent modulation of the stress response (Fig. 2.4 - 2.6). These findings suggest that protection of the gill integrity was achieved by varying underlying regulatory mechanisms, dependent on the severity of the insult.

Thereby, environmental hypercapnia can affect transcription by different means: An increase in the concentration of carbon dioxide, as well as the accumulation of bicarbonate in order to buffer the intracellular pH, result in a new steady-state equilibrium between CO_2, HCO^{3-} and H^+ (Ishimatsu et al. 2004, Pörtner et al. 2004). These changes in intracellular chemistry can influence several signalling cascades and, ultimately, transcription factors by various means. For example, soluble adenylate cyclase (sAC) is an enzyme that catalyses the synthesis of cAMP, which in turn exerts regulatory functions on a vast range of biochemical pathways (Zippin et al 2001). Though cAMP can be produced by additional sources, sAC has been demonstrated to directly respond to shifts in the CO_2/HCO^{3-} ratio (Buck & Levin 2011**,** Tresguerres et al. 2011**,** Zippin et al. 2001) and is also involved in the acid/base regulation (Tresguerres et al. 2010).

In addition, hypercapnic conditions are known to induce oxidative and nitrosative stress (Dean 2010). Especially a reaction between CO_2 and peroxynitrite ($ONOO^-$) prevalently occurs in CO_2/HCO^{3-} buffered systems, subsequently leading to a reinforced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Dean 2010). Thereby, the magnitude and amplitude of ROS/RNS increase is relevant for adjacent processes (Dröge 2002): In addition to a baseline level, which

represents the byproduct of general metabolism, a mild to moderate elevation of these highly reactive molecules may provoke a regulatory imbalance, which affects redox-responsive signalling pathways, which in turn control multiple physiological functions including the activity of a vast range of transcription factors. A pronounced increase of ROS/RNS beyond a critical value, however, may elicit oxidative/nitrosative stress, which impairs enzyme functions and fosters lipid peroxidation processes. Two transcription factors, AP-1 and NF-*κ*B, both central in the mediation of stress- and inflammatory responses, are suspected to belong to the set of regulatory elements stimulated by ROS (Li & Stark 2002, Karin et al. 2001), though data appears to be conflicting and dependent on the species and tissue under investigation (Karin et al. 2001, Bowie & O'Neill 2000). Nevertheless, as described in detail in the following sections, the majority of genes investigated in this study indeed appear to be connected to elevated ROS concentrations or the actions of associated transcription factors.

General Response to Hypercapnia

As indicated by the combined increase in IL-1b, LMP7 and Grim19 mRNA concentrations in gills exposed to both, the medium and high treatment, a general response to environmental hypercapnia above a certain threshold seem to involve activation of innate immune parameters. As in other vertebrates, fish Il-1b is a cytokine and prominent key regulator of inflammation (Whyte 2007). Studies in isolated cells demonstrated that a ROS-induced imbalance in the redox state of a cell fosters the release of pro-inflammatory cytokines, such as IL-1b, in a dose-dependent manner (Haddad 2002). Reliant on the cell type under investigation, IL-1b in turn bears the potential to enhance ROS generation as well, thereby reinforcing any effects on redox-sensitive signalling cascades (Schoonbroodt & Piette 2000). If an increase in IL-1b mRNA translates into enhanced activity of this cytokine, downstream effects would comprise pronounced alterations in multiple other genes: The enzyme is known to promote the activity of the redox-sensitive transcription factor NF-kß (Kassahn et al. 2009, Sun et al. 2008) and further sensitizes the transcription machinery to cAMP by activating the cAMP-responsive binding element (CREB) of certain target genes (Sun et al. 2008). Thus, up-regulation of IL-1b gene expression

may indicate a general, yet vital response to hypercapnia as it vastly influences the cellular transcription machinery.

LMP7, also elevated in its expression in both treatment groups, is recognized as an inducible component of the immune-proteasomes (Kotamraju et al. 2006). Proteasomes possess catabolic functions by disintegrating molecules, such as enzymes, into their building elements (which can be recycled in anabolic processes). Hence, these cellular components are part of the protein quality control machinery (Bukau et al. 2006) and further possess cyto-protective functions (Stangl & Stangl 2010, Kotamraju et al. 2006). LMP7 transcription is enhanced by cAMP (Stangl & Stangl 2010), thus, providing a link to the influence of IL-1ß and Hypercapnia itself. Further, proteasome activity contributes to the activation of NF-kß by degrading its inhibitor, ik-ßa (Haddad 2002).

The third gene apparently involved in a general response to chronic hypercapnia was Grim19. Initially recognized as a regulator of apoptosis, recent studies exposed the pleiotropic nature of this enzyme, describing additional functions in the innate immune response as well as in the control of somatic growth and the activity of certain serin-proteases and caspases (Nallar et al. 2008). In addition, Grim19 has also been proofed to be essential for the assembly and integrity of the mitochondrial complex I of the electron transport chain (Huang et al. 2004). The enzymes' concentrations were also enhanced in smoker´s lungs as well as in ischaemic rat brain (Nallar et al. 2008), indicating an involvement in the processes triggered to balance effects of elevated reactive oxygen (and nitrogen) species. In general, ROS induced mitochondrial damage is considered a main driver in the development of pathologies (Murphy 2009). Thereby, Grim19 may be of special importance in the protection of gill integrity.

Overall, the cooperative up-regulation of this genes in both, medium and high, appear to reflect the response to altered cellular redox states, thus implying elevated formation of reactive molecule species as main cause for shifts in gene expression.

<u>Dose-dependent modulation of gene expression</u>

In the medium CO_2 treatment, individuals displayed an additional up-regulation of PRDX and Akirin. The former is part of a superfamily of enzymes, predominantly known for their anti-oxidant function (Perkins et al. 2015). Under

acute respiratory distress syndrome (which can also be caused by chronic lung infections and low pH values), PRDX1 has also been demonstrated to promote inflammation in the lungs of rats by enhancing the expression of several cytokines (Liu et al. 2014). Nevertheless, in the light of the histological data presented in this study, a likewise effect in the gills of turbot seems less likely. An increase in mRNA may therefore contribute to the protection of the cellular interior by boosting the scavenging of excess ROS, which may also help to fine-tune shifts in redox-signalling cascades by keeping concentrations of these highly reactive molecules below critical threshold.

Akirin, the other gene exclusively up-regulated under medium CO_2 concentrations, is known to influence the transcription of multiple immune-related genes by modulating the target specificity of Nf-KB (Bonnay et al. 2014**,** Goto et al. 2008). In Atlantic salmon (*Salmo salar*), Akirin2 has further been suggested to be involved in muscle differentiation (Macqueen et al. 2010). Consequently, the parallel up-regulation of PRDX and Akirin in the medium treatment seems to be part of additional protective measures, aiming to keep the cellular redox state within the borders of a regulatory imbalance. Further, the influence of both genes on signal transduction and transcription strongly suggest that dose-dependent modulations of cellular biochemistry are pronounced and may include pathways not represented by the genes applied in this study.

In the gills of turbot exposed to the highest treatment level, DFAD as well as ApoE displayed an increase in transcriptome abundance. The latter synthesizes Apolipoprotein E, a structural component in lipoproteins, which, alongside ApoB and ApoC, are the dominant compartment in very-low-density lipoproteins (VDLDs) (Tocher 2003). In turbot, its expression has been shown to link to the distribution of yolk-lipids to other organs during early development (Poupard et al. 2000). Similarly, elevated transcriptional abundance under extreme hypercapnia may indicate boosted exchange of lipids and proteins between different cell types of the gill tissue.

DFAD encodes delta-6 fatty acid desaturase, which catalyses the introduction of a double bond in several 18C PUFA pre-coursers, marking the initial step of HUFA synthesis (Tocher 2003). This gene has been detected in multiple fish species in various organs, including the gills. Studies indicate that an increase in the expression of respective genes indeed entails the production of LC-PUFAs (Zheng et al. 2005, 2004). However, carnivorous teleosts, such as the turbot, usually display low

capacities for actual HUFA synthesis (Vagner & Santigosa 2011). Nevertheless, DFAD is suspected to be stimulated by unfavourable environmental conditions as its synthesis appears to be fostered by low temperatures and salinities in order to maintain membrane fluidity (Vagner & Santigosa 2011). It has also been speculated that hypercapnia elicits modulations of the mitochondrial membrane fatty acid compositions as part of required metabolic adjustments (Heuer & Grosell 2014). Thus, an up-regulation of DFAD in this study may reflect both, a protective measure to counter increased rates of ROS induced lipid-oxidations, as well as mitochondrial adjustments to an altered metabolism. In the latter case, this finding would further cement the impression of a fundamentally different biochemistry in the highest tested treatment level. However, the purpose of DFAD up-regulation remains far from resolved and is most likely of ambiguous nature. Hence, for a better understanding, more scientific research is required. Nevertheless, the simultaneous increase in both, ApoE and DFAD transcript abundances, may indicate that intracellular ROS concentrations have reached a harmful level and require the execution of additional allocation of resources and repair processes.

In contrast to the suggested increase in fatty acid synthesis, the parallel reduction of COX, EF1α and STAT2 mRNA may indicate declined energy availability. COX encodes the subunit 1 of the cytochrome c oxidase, the protein complex, which marks the endpoint of the mitochondrial respiratory chain. Due to its vital role in aerobic respiration, this enzyme is considered a general indicator for metabolic activity (Frick et al. 2008, Nogueira et al. 2001).

The impression of partially suppressed metabolism is supported by the accompanied reduction of EF1α and STAT2 expression. STAT2 belongs to a family of transducer and activator of transcription (STAT) proteins and is predominantly involved in the expression-regulation of genes related to the innate immune system (Au-Yeung et al. 2013). It exerts its diverse effects by dimerization with different types of other regulatory elements. EF1α is involved in the eukaryotic translation machinery and has also been associated with other processes, such as proteolysis, apoptosis and viral propagation (Sasikumar et al. 2012). The protein´s action is regulated by several post-translational modulations (Lamberti et al. 2004), which is why the exact effects of an implied decrease in EF1α remain unknown. However, data suggests the abatement of the synthesis of an indefinite fraction of proteins, which further supports the assumption of suppressed metabolic activity.

Hypercapnia induced reduction of metabolism and adjacent suppression of protein synthesis has been described for various aquatic taxa, though this phenomenon is not a general response and depends also on factors such as carbon dioxide concentration, species and tissue under investigation (Fabry et al. 2008**,** Widdicombe & Spicer 2008, Seibel & Walsh 2003). While often found in conjunction with uncompensated acidosis, a disruption of the organism's ability to direct sufficient oxygen to the tissues seem to constitute the primary basis for depressed metabolic activities and damped protein synthesis (Fabry et al. 2008, Widdicombe & Spicer 2008, Seibel & Walsh 2003). Indeed, turbot subjected to the high treatment displayed reduced oxygen consumption rates as well as diminished concentrations of blood haemoglobin (Stiller et al. 2015).

However, measurements of oxygen consumption rates were conducted on a weekly basis, thus, provide a rather low temporal resolution. Due to the strong indication of ROS activities, it is therefore possible that molecular mechanisms, detrimental to the integrity of the mitochondria, may contribute to the implied reduction of metabolic activity: Uncompensated oxidative stress bears the potential to impair functionality of cellular components involved in the up-take and processing of oxygen due to general uncontrolled reactions with lipids and enzymes. Further, it is known that the transcription machinery of the mitochondria is less efficient in comparison to the nucleus and, therefore, more prone to deleterious ROS activities (Cline 2012).

Taken together, gene expression data obtained in this study provides evidence for increasing intracellular hypoxia and oxidative stress in the gills of turbot, dependent on the severeness of environmental hypercapnia. The implied progressive shift towards anaerobic metabolism would not be contradicted by the up-regulation of genes such as DFAD, IL-1ß or Grim19, as cells have been shown to sustain the transcription (and translation) of genes required for coping and damage repair, even under anoxic conditions (Storey & Storey 2004). However, though the picture provided by our data is relative coherent, more research, including high throughput molecular data and actual assessment of ROS concentrations, is required to confirm these preliminary conclusions.

Further Implications and Outlook

Interestingly, reduced metabolic activity and the elicitation of oxidative stress have also been described as symptoms occurring at a progressive rate the more an ectotherm species gets driven away from its temperature optimum. According to the so called concept of oxygen- and capacity limited thermal tolerance, temperatures beyond pejus cause an accretive disruption of ventilation and oxygen transport to the tissues, resulting in an increasing mismatch between oxygen demand and supply (Pörtner 2010, Pörtner & Lannig 2009, Pörtner & Knust 2007, Pörtner 2002). If adjustment processes fail to maintain aerobic scope, the ensuing shift towards an anaerobic mode of energy generation gives rise to both, mitochondria-generate ROS as well as declining metabolic activity. On the organismal level, the resulting impaired performance capacity sets physiological constraints, which become more and more perceivable and ultimately prevent a species to survive outside its thermal tolerance range.

Expanding on this concept, Kassahn et al. (2009) suggested a model which generally aims to link physiological and transcriptional responses to stress: It states that not only temperature, but most stressors, despite different modes of action, ultimately disrupt the organism's oxygen balance and foster the generation of oxidative stress within the cells. If uncompensated, the joint effects of both factors then lay foundation for pathologies occurring at higher levels of biological organisation. Consequently, a core set of mediators common to all stress responses are considered to regulate processes required to maintain aerobic scope and performance capacity. Due to the infliction of oxidative stress, these mediators appear to predominantly comprise redox-sensitive signal transducers and transcription factors, such as NF-kß. Thus, the overall picture implied by all responsive target genes provides strong support for this concept. Based on temperature, the authors also combined stress response curves for cellular, physiological and organismal performance functions to distinguish between four main stages of animal condition, dependent on the degree of deviation from a theoretical optimum (Fig. 2 in Kassahn et al. 2009). Given the central role of the gills in adjusting to the tested conditions, the application of this principal may allow to infer possible implications for the whole animal's future survival and resistance to additional stress under the conditions tested in our study. Thus, with an applied carbon dioxide concentration

considerably higher than in the turbot's natural habitat, it is likely that fish exposed to our lowest treatment level already reside beyond their optimal range (according to Kassahn et al. (2009), beyond the so called pejus value Cp). Hence, while successfully able to maintain aerobic scope and therefore capacity to cope with the stressor, energy allocation in favour of physiological adjustments, for example in the oxygen delivery system, may translate in slightly reduced overall performance when compared with animals in common sea water.

Yet, while the lack of a real control renders the classification of fish subjected to 3000 µatm mg L^{-1} CO_2 difficult, indications for increased cellular ROS concentrations in the other two treatment groups clearly suggest the passing of Cp. The additional indication of enhanced anti-oxidant activity and transcriptional regulation in gills dissected from individuals exposed to 15000 µatm mg L^{-1} carbon dioxide betokens a condition in which comprised aerobic scope is countered by cellular anti-oxidant and repair mechanisms. While individuals may survive under these conditions, the vulnerability to additional stressors, such as pathogenes, would be increased. Whether this also means that a critical concentration (Cc) has already been exceeded, remains open. In contrast, insinuations of severe oxidative stress and reduced metabolic activity in the gills of fish exposed to 25000 µatm mg L^{-1} CO_2 likely reflect the impact of a carbon dioxide concentration, which not only has exceeded a critical, but maybe even a detrimental point (Cd). In this state, with the molecular stress response already at the brink of its capacity to maintain cellular functionality, survival is considered to be temporary and additional stress would accelerate death. This rough classification of treatment groups is supported by the physiological data presented by Stiller et al. (2015), in which a gradual, dose-dependent decline of performance traits, such as the specific growth rate, is demonstrated.

This study provides transcriptional evidence for the central role of ROS (and possibly RNS) in the regulation of cellular biochemistry under chronic, severe hypercapnia in the gills of a marine teleost. Thereby, gene expression data revealed details of the stress response, which would not have been noticed by the investigation of histological gill traits alone. In this regard, several target genes, for example COX, may proof to be a useful marker for the determination of intracellular hypoxia and, to an extent, a condition beyond a detrimental threshold.

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CHAPTER III

Differential gene expression in Atlantic cod (Gadus morhua) larvae under future ocean acidification

Abstract

While much research effort has been dedicated to examine the impact of ocean acidification (OA) on marine organisms, information on commercial fish species is still widely lacking. In Atlantic cod (*Gadus morhua*) larvae, exposure to elevated levels of carbon dioxide has been found to cause several pathologies, with the severity of the symptoms increasing with the level of pCO_2. To illuminate the underlying mechanisms involved in the response to OA, we reared newly fertilized eggs of the Atlantic cod for two months under three different pCO_2 regimes: 380 µatm (control), 1800 µatm (medium) and 4200 µatm (high) in mesocosms. We extracted RNA from whole fish sampled during larval (32 dph) and early juvenile stage (46 dph) for relative expression analysis of 19 different genes related to metabolic activity. At 32 dph, we detected a dose-dependent response to different levels of pCO_2: 1) fish subjected to the medium treatment displayed an up-regulation of fat and muscle associated glycogen synthase (gys1), fatty acid synthase (FAS), acetyl-coenzyme A carboxylase (ACoA), peroxisome proliferator-activated receptor beta (PPARβ) and carnitine palmitoyltransferase I (CPT1a). These findings indicate tissue specific adaptations to environmental hypercapnia, as well as costly, yet successful compensatory stress response. 2) larvae exposed to the highest pCO_2 treatment shared a comparable up-regulation of AcoA and PPARβ, with an additional increase in 6-phosphogluconolactonase (6PGL), suggesting oxidative stress and a deregulated metabolism. At 46 dph, no significant differences in gene expression were detected, confirming a higher resilience of early juveniles in comparison to metamorphic larvae when exposed to elevated pCO_2 up to a concentration of 4200 µatm.

Introduction

Atmospheric CO_2 levels are currently rising at a rate 100 times faster than seen in the past 650,000 years, largely driven by anthropogenic activities such as burning of fossil fuels and changes in land-use. As the oceans are in continuous balance with the atmosphere, approximately half of the CO_2 in the atmosphere will be absorbed by the oceans, leading to an estimated drop in pH of 0.4 units by the year 2100 (Pachauri et al. 2014, Ilyina et al. 2013, Solomon et al. 2007, Caldeira & Wickett 2003). Locally, the effects can be much more pronounced especially in coastal regions with upwelling of oxygen poor, CO_2 rich water, and some organisms are already experiencing pCO_2 values not predicted for another 100 years (Sasse et al. 2015, Hofmann et al. 2011). Early life stages of marine organisms have been shown to be especially susceptible to environmental changes, and effects of ocean acidification on marine fish larvae have been documented regarding development (Frommel et al. 2014, 2012, Baumann et al. 2012, Munday et al. 2009), RNA/DNA ratios (Franke and Clemmesen 2011), behaviour (Forsgren et al. 2013, Nilsson et al. 2012, Ferrari et al. 2011), and otolith growth (Maneja et al. 2013, Munday et al. 2011, Checkley et al. 2009).

Adult fishes can control their acid-base balance by bicarbonate buffering, mainly across the gills, and thus compensate for changes in ion concentrations as caused by ocean acidification. However, in the early life stages, the hypercapnia-induced increase in protons can exceed the ion-regulative capacity, resulting in an uncompensated acidification within the animal (Heuer and Grosell, 2014). Even if the individual is able to cope with the stressors, the necessary response may require energy, which comes at a cost of other processes (Deigweiher et al. 2010, 2008, Melzner et al. 2009).

The Atlantic cod, *Gadus morhua*, has a wide distribution throughout the North Atlantic. The Norwegian coastal cod population used in this study live and spawn in a large number of fjords along the entire Norwegian coast and near the Lofoten Islands (Nordeide, 1998). These high latitudes are assumed to be particularly impacted by future ocean acidification, due to higher solubility of CO_2 in cold water, high primary productivity and melting of sea ice (Fabry et al. 2009, Steinacher et al. 2009, Bellerby et al. 2005), and pH values are predicted to approach 7.7 over most of the coastal

Arctic Ocean by the year 2100 (Denman et al. 2011) with a 6-fold amplification in seasonal CO_2 variation (Biastoch et al. 2011).

In a preceding study, Frommel et al. (2012) simulated different ocean acidification scenarios by exposing larvae of Norwegian coastal cod to three levels of pCO_2 (control: 380 µatm, medium: 1800 µatm and high: 4200 µatm) from newly fertilized eggs to seven weeks post-hatch. Several pathologies occurring in larvae subjected to elevated carbon dioxide were observed, with symptoms peaking at 32 days post hatch (dph). These symptoms comprised increased growth, accumulation of lipids throughout the body as well as severe tissue damage in multiple organs, increasing with carbon dioxide concentration. While these data implied severe stress and a disturbed metabolism as a consequence of elevated CO_2 during a crucial phase in the development of cod, several questions regarding the underlying molecular mechanisms remained unanswered. To address this issue, we extracted RNA from whole larvae collected during the original experiment at 32 and 46 dph and analysed the mRNA expression of 19 key enzymes involved in the regulation of major biochemical pathways. In light of the reported lipid accumulation and tissue damage, the application of individuals which have been subjected to the exact same experimental set-up gave us the unique opportunity to investigate transcriptional adjustments in the context of a failed stress response. Our aim was to gain deeper insight into the assumed disruption of general metabolism and to further elucidate the main causes of the observed pathologies. In addition, we wanted to test whether cod were able to cope with these conditions at a later developmental stage, as suggested by physiological data, or whether there were signs of continuous stress or carry-over effects on the molecular level. Finally, we screened our results for potentially valuable biomarkers for future studies related to environmental stress.

Methods

Larval Rearing and Sampling

Newly fertilized eggs of Norwegian costal cod were obtained from the hatchery in Parisvatten (Norway) and reared for 2 ½ months in nine land-based mesocosms at the University of Bergen's experimental marine facility in Espegrend, Norway. The 2300-L mesocosms were set in a flow-through system with water taken

directly out of the Bergen Fjord, allowing for natural conditions such as temperature, salinity and water quality, in addition to the natural light cycle and intensity. The eggs were incubated in floating buckets with mesh bottoms (allowing for free gas exchange) inside the mesocosms. After hatch, a set number of larvae were counted out of the buckets into the mesocosms starting out each tank with the same number of larvae. Natural food was provided daily by filtering seawater from the fjord over three different mesh sizes and concentrating the middle fraction in a large plankton tank. With this method, the size spectrum of the plankton could be adjusted to the size of the larvae during the course of the experiment, allowing them to receive the optimal prey. Each day, a subsample of the zooplankton (zpl) remaining in each mesocosm was counted out and the prey volume fed daily was adjusted to ensure equal food densities (2000 zpl L^{-1}) in all tanks. The tanks were aerated with a fine bubble ring and oxygen levels were monitored daily, along with temperature, salinity and pH.

Three pH levels, control (pH=8.2), medium (pH=7.5) and high (pH=7.1) over three replicates were maintained in the mesocosms by dissolving carbon dioxide (CO_2) into the water with fine diffusers. The addition of CO_2 was controlled by two mobile computers (Aquastar© by iks) by opening and closing individual magnet valves connected to a CO_2 cylinder and the diffusers in the tanks. The pH in each of the tanks was measured every 15 minutes and adjusted to the set level of pH accordingly. By connecting the CO_2 diffuser to the inflow at the bottom of the tank in addition to the air bubble ring, rapid mixing and a homogeneous water pH was assured. In addition to the pH measurements from the system, the pH was measured daily with a laboratory-grade glass pH probe (WTW) calibrated with a seawater standard and checked with the Dickson standard. Weekly water samples were taken for dissolved inorganic carbon (DIC) and total alkalinity (TA) measurements for calculations of pCO_2 levels in the tanks. The CO_2 concentrations corresponding to the pH levels were estimated to be 1800ppm CO_2 at a pH of 7.5 and 4000ppm CO_2 at a pH of 7.1, while the control was left to fluctuate freely around 380 µatm at a pH around 8.2, without manipulation but with close monitoring. The water temperature increased from 5°C to 10°C during the experimental period of 10 weeks while the salinity remained at ambient levels between 33 and 34. Oxygen was measured daily with a hand-held oxygen device (WTW Oxi 340i) and remained above 90% saturation.

Fish at 32 dph were sampled using a cylinder spanning the depth of the mesocosm in order to sample larvae randomly at all depths. At 46 dph, faster larvae were able to avoid the cylinder and the fish had to be sampled using large plastic ladles. Sampled larvae were preserved in RNA later and stored at -80°C.

Table 3.1: Genes under investigation in this study.

Gene	Function	Primer sequence	Slope	Efficiency
ACoA	Fatty acid synthesis	fw: CCATTCGCTTCGTCGTCAT rev:CGCCTTTGATGTACTCTCTGCAT	-2.86	2.24
CPT1A	Fatty acid synthesis	fw: CCTGACCAGCTACGCCAAGT rev:CTTATCAAACCACCGGTCGAA	-3.77	1.84
FAS	Fatty acid synthesis	fw: CCTGGCCATGAACGTGTTC rev:CTCGTTCAGATCCTGGTTGATG	-3.24	2.03
BAL	Digestion	fw: TCGCTGGTGCGAATGATATG rev: TGGTGGGCACGTCCAATC	-3.89	1.81
TRP1A	Digestion	fw: CTCTCCCACGCCGACTGT rev: CAGAACATGGACTGGGTGATCA	-3.65	1.88
Trypsinogen	Digestion	fw: TTCGCTGAGGAGGACAAGATC rev:TGGGCCTGGGAGTGCTT	-3.64	1.88
PPAR1b	Transcription factor	fw: CAGCACCTCGCCCTTTGT rev: CCGCTCTCCGCTTTCCAT	-3.25	2.03
PPAR1g	Transcription factor	fw: CTCCTTCCCCAGGGTGATG rev: GACACTCGATGTTCAGCGTGAT	-3.25	2.03
GH	Growth hormone	fw: TCCATCACATCACACGATCAAG rev: CATCAACGCCAGCAACACA	-3.20	2.05
GAPDH	Glycolysis	fw: AGGTGGTGTCCACAGATTTCAA rev: TGCCAGCACCAGCATCAA	-3.25	2.03
GDH	Link b/t catabolic and metabolic pw	fw: CCACCGGTCGTGGTGTGT rev: GTGTATGACGCTTCCTGGATGA	-3.15	2.07
GYS1	glycogen synthesis	fw: TCGTGCAACCAGCTCACTTC rew: GCGCCGGCTCTGCTT	-3.98	1.78
GYS2	glycogen synthesis	fw: TCCGTATGTGGAGAGCTAACGT rev:GGTTGGTGGGCTCGATGA	-3.45	1.95
PepCK	Gluconeogenesis	fw: GAACACAAAGGTAAAGAGATCATGCA rev:GACAGGTAGTGGCCGAAGTTG	-2.91	2.21
6PGL	Pentose-phosphate pathway	fw: GCCGACAGATATTTCCAGCATT rev: CGCTCTGCTTCCTGCTGATAA	-3.40	1.97
NaK	Acid-Base regulation	fw: ACGCAGAAAGATCGTAGAGTTTACTTG rew: ACTGGACCACCACGATACTG	-3.25	2.03
Ubi	Housekeeping gene	fw: GGCGTACCCTCTCGGATTACA rev: AGACGGAGCACCAGATGCA	-3.20	2.05

RNA extraction, assay development and gene expression analysis

Whole fish were thoroughly homogenized using a vortex mixer and zirconia beads. Afterwards, total RNA was extracted using Invitrap® Spin Tissue RNA Mini Kit (Stratec, Berlin, Germany) according to the manufacturers' instructions. The amount of total RNA was assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). An exemplary number of samples (30%) were analysed via the Experion® System (Biorad, Munich, Germany), to test for RNA integrity. All samples yielded a RQI of 9.0 and higher and were therefore considered suitable for further gene expression analysis. Genomic DNA was eliminated from the samples by DNase treatment according to the manufacturer's description (Ambion, Austin, TX, USA). The RNA was then stored at –80°C until further processing.

To detect biochemical aberrations, which may provide an explanation for the accumulation of lipid droplets and tissue damage, nineteen genes, representative for the activity and regulation of the most essential metabolic pathways, were picked for screening. Gene names, symbols and their function are shown in Table 3.1. The set comprised 6-phosphogluconolactonase (6PGL), citrate synthase (CS), sodium-potassium pump (NaK), phosphoenolpyruvate carboxykinase (PepCK), glyceraldehyde-3phosphate dehydrogenase (GAPDH), glutamate dehydrogenase (GDH), growth hormone (GH), acyl-Coenzyme A dehydrogenase (AcylCoADH), acidic ribosomal protein (ARP), fatty acid synthase (FAS), acyl-coenzyme A carboxylase (ACoA), carnitine palmitoyltransferase 1A (liver) (CPT1α), bile salt-activated lipase (BAL), trypsinogen (TRP1α), glycogen synthase 1 and 2 (GYS1 and GYS2), peroxisome proliferator-activated receptor beta and gamma (PPARβ and PPAR1ɣ) and ubiquitin (Ubi).

Primer design was based on the data set of the published large-scale Atlantic cod sequencing effort (Star et al. 2011, accession number: CAEA000000000.2). Gene annotations were originally done with an automatic pipeline, but annotation of the studied genes was also re-evaluated manually. The primer pairs amplify PCR products between 69-134 base pairs (bp). Primer efficiencies were evaluated based on triplicates of two-fold serial dilutions, comprising six steps (1000 - 31 ng of total RNA input).

cDNA synthesis was conducted using TaqMan Reverse Transcription Reagent containing Multiscribe Reverse Transcriptase (50 U/µl) (Applied Biosystems, Foster City, CA, USA). For each reaction, 500 ng of total RNA input was used. In addition, no-template control (ntc) and RT-control (a duplicate RNA sample analysis where only the reverse transcriptase enzyme is left out) reactions were included for quality assessment. RT-controls were not performed for every individual sample, but were run for each assay or gene, with the same sample as used to make the dilution curves on the 96 well plates. Reverse transcription was performed at 48°C for 60 min by using oligo dT primers (2.5 µM) for all genes in 20 µl total volume. The final concentration of the other chemicals in each RT reaction was: MgCl2 (5.5 mM), dNTP (500 mM of each), 10X TaqMan RT buffer (1X), RNase inhibitor (0.4 U/µl) and Multiscribe reverse transcriptase (1.67 U/µl).

2.0 µl cDNA from each RT reaction for all genes was transferred to a new 96-well reaction plate and the real-time PCR run in 20 µl reactions on the ABI 7500 Real-Time PCR system (life technologies, Darmstadt, Germany). Real-time PCR was performed using the SYBR Green Master Mix (life technologies, Darmstadt, Germany), which contains FastStart DNA polymerase and gene specific primers (500 nM). PCR was achieved with initial denaturation and enzyme activation for 5 min at 95°C, followed by 40 cycles of 10 s denaturation at 95°C, 20 s annealing at 60°C and 30 s elongation at 72°C.

Data Analysis

To identify the most suitable reference genes, the expression stability of all 19 candidates was evaluated by applying the *NormFinder* applet for Microsoft Excel (Anderson et al. 2004). Beforehand, the Ct values were transformed to quantities using gene specific efficiencies. *NormFinder* calculates a gene-stability value with a mathematical model based on separate analysis of the sample subgroups and estimation of both intra- and intergroup variation in expression levels (Andersen et al. 2004). Arp and Ubi were determined as the most stable pair of genes (M-Value 0.182) and therefore applied as reference to calculate normalized relative quantities (NRQs). NRQs were calculated based on the formula presented by Hellemanns et al. (2007), in which assay specific amplification efficiencies are considered:

$$NRQ = \frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[f]{\prod_0^f E_{ref_0}^{\Delta Ct,ref_0}}}$$

To calibrate against a common baseline, the average crossing point for a given gene across all samples was applied.

All statistics were conducted within an R environment (R Core Team, 2016). Uniform Box-Cox transformations were applied to convert NRQs to become normally distributed. Assumptions of normality and homogeneity of variance were checked using Shapiro-Wilk-Test and Fligner-Killeen-Test. To test for overall treatment effects of hypercapnia and larval development on the transcriptome level a type III two-factorial MANOVA was applied, followed by a Pillai´s Trace test. Afterwards, treatment effects on single genes were tested via univariate ANOVAs. Here, specified contrasts were used to identify significant effects between groups at both time points sampled, as well as within groups between 32 dph and 46 dph.

Results

According to Pillai`s trace test, there was a significant effect of experimental CO_2 treatments ($p = 0.02$), larval development ($p = 0.001$) and an interaction of both factors ($p=0.015$) on overall gene expression patterns. Subsequent univariate ANOVAS found that ten out of the seventeen genes of interest were significantly up-regulated, either under elevated levels of carbon dioxide at 32 dph or between 32 dph and 46 dph, respectively.

At 32 dph, a group of genes, namely Gys1, PPARβ, CPT1α, AcoA and FAS were up-regulated in the medium treatment. Under a pCO_2 of 4200 µatm, the same applied for AcoA, 6PGL and PPARβ, whereas the latter curtly missed the significance threshold of 0.05 (Fig. 3.1). Between sampling points, transcript levels for GAPDH increased in all three groups, while enhanced mRNA concentrations of NAK, 6PGL, TRP1α, PPARβ and (almost) CPT1α where restricted to the control. Further, expression of FAS increased between 32 dph and 46 dph in individuals subjected to the control and the high treatment. The latter group also displayed an increase in BAL and Gys1 mRNA concentrations (Fig. 3.2).

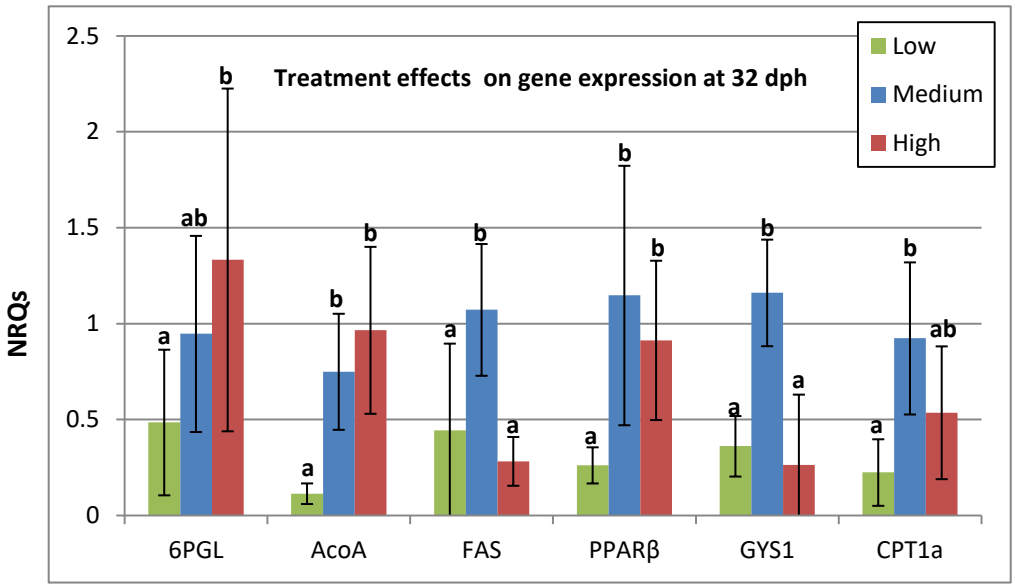

Figure 3.1: Genes yielding significantly different relative transcript quantities (P < 0.05) during late larval stage (32 dph) in response to different treatment levels: control (380 µatm), medium (1800 µatm) and high (4200 µatm). Only genes affected by the treatment are shown. Error bars indicate the standard 95% confidence interval.

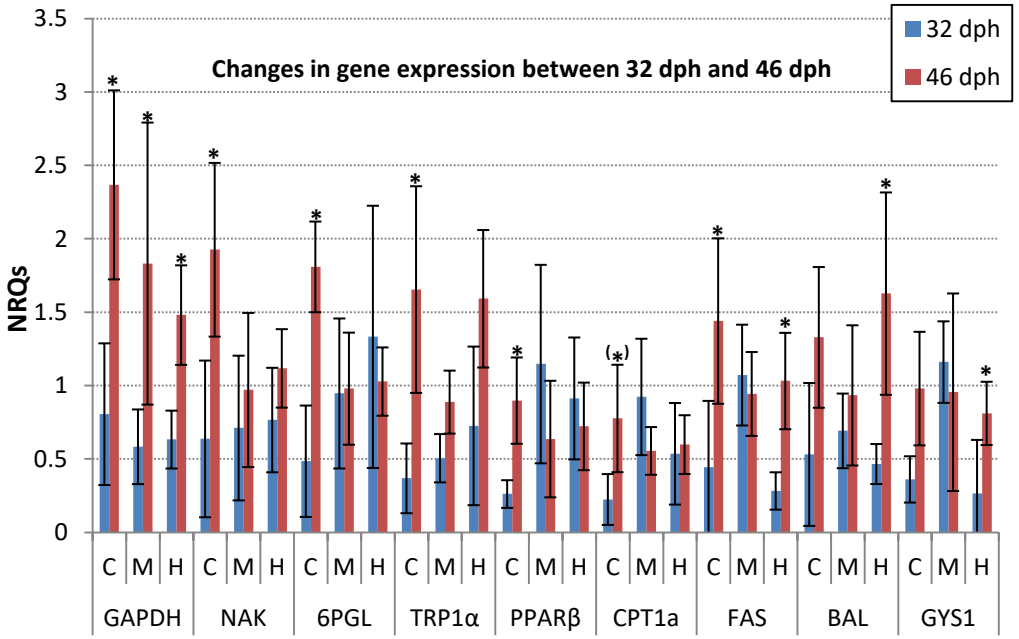

Figure 3.2: Genes differently expressed at 46 dph in comparison to 32 dph (P < 0.05): control (C), medium (M) and high (H). Significant results are marked with an asterisk.

Discussion

In this study, we demonstrated alterations on the transcript level in Atlantic cod larvae as a result of chronic exposure to elevated pCO_2 levels which can be compared to predicted end of next century ocean acidification (1800 µatm, Solomon et al. 2007, Caldeira & Wickett 2003) and extreme cases of coastal upwelling of oxygen-poor, CO_2-rich water (4200 µatm, Thomsen et al. 2010), respectively. Especially at 32 dph, the impact of the tested conditions on gene expression were most conspicuous, thus, supporting previous findings which marked this developmental stage as being responsive to environmental hypercapnia and its follow-up effects (Frommel et al. 2012). Here, our results demonstrated an increase in the expression of several genes connected to regulation of transcription, mobilization of energy and anabolic metabolism with a particular emphasis on glycogen and fatty acids synthesis (Fig. 3.1). In particular the latter aligns well with the reported hypercapnia induced formation of lipid droplets throughout the body, which has been reported for cod larvae subjected to the same experimental treatments (see Frommel et al. 2012). However, both treatment levels shared common response elements while also displaying dose-specific transcriptional adjustments. A second group of genes differed in expression between 32 dph and 46 dph, hence, indicating that elevated carbon dioxide concentrations potentially interfere with the temporal succession of transcription during larvae development (Fig. 3.2). This cluster also comprised several genes which already displayed enhanced mRNA transcript abundances at 32 dph. As we will discuss in the following sections, our findings may reflect varying degrees of oxidative stress and diminished metabolic capacity. Both phenomena could be ascribed to several localized consequences of environmental hypercapnia and subsequent disturbances of blood acid-base equilibrium.

<u>Hypercapnia induced local disruption of oxygen supply and lipid accumulation</u>

Since the formation of pathologies was most abundant in larvae subjected to 4200 µatm, it was somehow surprising to find that most affected genes at 32 dph were upregulated in the medium treatment. The up-regulation PPARβ, a member of the nuclear receptor superfamily of ligand-dependent transcription factors (PPARs) may indicate comprehensive changes in metabolism and cellular functions (Wagner &

Wagner 2010). Furthermore, gys1 encodes an isoform of glycogen synthase which is associated with fat and muscle tissue (Ferrer et al. 2003). In addition, cod larvae displayed an increase in AcoA and FAS transcripts. AcoA encodes Acetyl-Coenzyme A carboxylase, which catalyses the synthesis of malonyl-CoA from acetyl-CoA, a reaction which marks the first step of fatty acid synthesis. FAS is a multi-enzyme protein that catalyses the synthesis of palmitic acid. Finally, CPT1α, which encodes carnitine palmitoyltransferase 1A, enables β-oxidation by transporting fatty acids through the membranes into the mitochondria.

As fatty acid synthesis and β-oxidation are inversely regulated, the suggested boost of both metabolic pathways seems contradictory. Yet, as RNA has been extracted from whole cod larvae, this finding may simply reflect cell type or tissue specific adjustments to the tested conditions within the whole animal. Enhanced mobilization of energy, as suggested by CPT1α, may mirror a general response to hypercapnia (Wittmann & Pörtner 2013). On the opposite, elevated fatty acid synthesis, as well as other changes in cellular biochemistry suggested by our data, may link to mechanisms which could be associated with lipid droplet formation and tissue damage, two of the most conspicuous findings reported for cod larvae subjected to the same experimental treatments (see Frommel et al. 2012).

These pathological effects and potentially associated changes in gene expression are most likely the outcome of hypercapnia induced fluctuations in blood pH and oxygen transport. In general, adult fish can compensate pH imbalances efficiently after an initial respiratory acidosis within hours by increasing extracellular HCO_3^- and excreting excess protons, mainly across the gills. However, despite compensated pH, levels of pCO_2 and HCO_3^- are increased in the extracellular fluids, which can have severe downstream consequences for the organism (Heuer & Grosell, 2014). At 32 dph, cod larvae have reached a developmental stage characterized by an advanced, yet incomplete gill maturation (Hunt von Herbing 1999). Therefore, acid-base regulation at this stage is potentially not be fully efficient, yet. A contemporaneous gain in body surface may additionally cause increasing CO_2 influx. Alternatively, environmental hypercapnia may dampen the carbon dioxide gradient between the organisms interior and its sourrounding, resulting in a retention of high blood pCO_2 (Esbaugh et al. 2012). Irrespective of the cause, associated disruptions of the blood acid-base equilibrium may further entail difficulties in supplying sufficient oxygen to the cells.

HIF-1 is also known to stimulate the expression of glycogen synthase (gys): In human adipocytes, hypoxia has been shown to trigger glycogen synthesis, which indeed is mediated by an increase in gys mRNA (Leiherer et al. 2014, Pescador et al. 2010). It has been suggested that an elevated glycogen production provides cellular protection during phases of low oxygen tension, as it creates a substrate for glycolysis, the main energy source under these conditions (Leiherer et al. 2014, Windisch et al. 2011, Markan et al. 2010; Pescador et al. 2010). Consequently, the observed up-regulation of gys1 would be in line with the notion of an impeded oxygen supply at sides of lipid droplet formation. Thus, hypercapnia induced local hypoxia may provide the best explanation to link the implied increase in fatty acid and glycogen synthesis to the reported pathologies (see Frommel et al. 2012). This assumption is further supported by the identification of HIF homologues in fish, which seem to exert comparable regulatory control over transcription (Nikinmaa & Rees 2005).

Reduced capacity and oxidative stress as underlying mechanism for dose-dependent, transcriptional adjustments

While gene expression data and physiological observations align rather well in the medium group, our results are more difficult to explain at 4200 µatm CO_2. As mentioned beforehand, this carbon dioxide concentration reflects a possible, yet extreme scenario, which can be caused by costal upwelling events (Thomsen et al. 2010). Under these conditions, only a fraction of genes (namely AcoA and PPARβ) displayed an increase in transcript abundances comparable to those observed under moderate carbon dioxide concentrations. In addition, 6PGL, an enzyme associated with the oxidative phase of the pentose phosphate shunt, was significantly up-regulated in comparison to the control. In consideration of the reported pathologies, which were most prominent at high pCO_2 levels, respective changes in gene expression may reflect a further deteriorated and potentially uncompensated disruption of cellular homeostasis.

As FAS transcripts remained on a level similar to that of the control group, an increase in AcoA expression could rather indicate a net gain in Malonyl-CoA than in fatty acids. This, in turn, could result in cytotoxic effects - either by promoting apoptosis as shown in cancer cells (Pizer et al. 2000) or by impeding oxidation of fatty

acids by inhibiting CPT1α (Saggerson 2008), thereby contributing to the accumulation of lipids. The latter mechanism may be of special relevance under conditions of hampered oxygen supply, which has been demonstrated to increase the uptake of free fatty acids in cardiac myocytes of male wistar rats due to a hypoxia-induced translocation of fatty acid transporters (Chabowski et al. 2006). However, a functional explanation for a potential shift in the AcoA/FAS ratio and a subsequent increase of Malonyl-CoA remains elusive.

Enhanced activity of the pentose phosphate shunt, as suggested by elevated concentrations of 6PGL mRNA, could be linked to the abnormal deposition of lipids in cod larvae, too. However, a raised need for NADPH may also hint at oxidative stress because of its requirement in the reduction of glutathione disulphide to glutathione, an integral element of the anti-oxidant defence. This notion would be further supported by a simultaneous increase in PPARβ expression, though results failed to cross the significance threshold and, thus, should rather be seen as a strong tendency. Yet, this transcription factor has been demonstrated to participate in the interception of apoptosis and oxidative stress (Grimaldi 2010, Jiang et al 2009).

The deposition and accumulation of fat in cells which are not specialized adipocytes can promote lipotoxicity, which in turn is considered to mediate its detrimental effects by promoting oxidative stress: An overabundance of palmitate and other saturated fatty acids can enhance ROS concentrations by stimulating an inflammatory response (Milanski et al. 2009, Ajuwon & Spurlock 2005) or by inducing endoplasmic reticulum stress (ER stress, Han & Kaufman 2016, Brookheart et at. 2009). ER stress, in turn may result in oxidative stress as a side effect of protein oxidation (Malhotra & Kaufman 2007) or Ca^{2+} mediated interference of the mitochondrial electron transport chain (Csordás & Hajnóczky 2009, Zhang & Kaufman 2008). ER stress, oxidative stress and inflammation are interconnected and usually occur in concert, whereas cell type, stressor and other factors determine the relative due of each process to the stress induced phenotype (Zhang 2010). However, under conditions of fatty acid overload, ER stress and ensuing mitochondrial dysfunction appear to be a pivotal route in the promotion of ROS synthesis and a disrupted oxidative metabolism (Egnatchik et al. 2014, Brookheart et al. 2009). In addition, oxidative stress can be further intensified by hypercapnia itself, in particular in bicarbonate buffered systems. Carbon dioxide can react with peroxynitrite and, thus, generate several more reactive oxygen and nitrogen species (Dean 2010). A

pronounced shift towards an oxidizing cellular milieu and potential ensuing apoptosis could also provide an explanation for tissue damages reported by Frommel et al. (2012).

Regardless of uncertainties concerning the complex details which underlie the hypercapnia induced formation of ROS, increasing oxidative stress alongside increasing environmental carbon dioxide concentrations would be in line with a stress concept proposed by Kassahn et al. (2009). This model suggests that the effects of most stressors converge on increasing concentrations of reactive oxygen species and a narrowed capacity for aerobic respiration, thus, providing a framework which offers a mechanistic link between gene expression and physiological data. Consequently, both factors outlined by Kassahn's model of capacity limitation may differ between treatment levels in their respective intensity and, therefore, explain specific transcriptional adjustments. Derived from our data, accretive lipotoxicity and an increasing difficulty to transport and utilize oxygen appear to be the main forces which advance these parameters. Increased ROS levels above baseline could also explain certain commonalities in the transcriptional stress response. For example, increased expression of PPARβ transcripts at 1800 µatm pCO_2 may have similar protective connotations, though intracellular ROS concentrations at this stress intensity would likely be less severe.

Interaction between CO_2 and larval development

Frommel et al. (2012) reported a considerable dwindling of both, severeness and abundance of pathologies in 46 dph in comparison to 32 dph. Further, though some individuals still dealt with injured tissue, control and treatment groups were not significantly different anymore. In agreement with this observation, no significant differences in gene expression could be detected either, implying that early juveniles at this stage were no longer affected by environmental hypercapnia. In a recent publication, Stiasny et al. (2016) reported a doubling of daily mortality rates in Atlantic cod larvae exposed to a setting comparable to this study (~1100 µatm pCO_2). This finding was consistent across two different cod populations and was calculated to result in a decrease of stock recruitments down to 8% and 24%, respectively. Thus, given the even higher concentrations tested in our experiment, selective mortality

between 32 dph and 46 dph in the treatment groups may provide a likely explanation for an approximation of both, larvae conditions and gene expression profiles.

However, several genes were found to change in their respective transcript abundances between 32 dph and 46 dph (Fig. 3.2). Among this group of genes, only GAPDH displayed a similar increase across all three tested conditions, suggesting a general gain in glycolysis activity. This may simply connect to the accumulation of muscle tissue during growth, progressively overlying the transcript patterns of undifferentiated soft tissue. The remaining accretions of mRNA were restricted to the control, the highest tested treatment level or both. Yet, due to the lack of physiological data, this outcomes are difficult to interpret. Nevertheless, when contrasted against the control, hypercapnia seems to prevent an increase in transcript abundances of genes like 6PGL (at medium carbon dioxide concentrations), NAK or TRP1α.

Trypsin is an important proteolytic enzyme, crucial for the nutrition and development of fish larvae (Gudmundsdóttir & Pálsdóttir 2005). In cod, its inactive precursor trypsinogen is synthesized in the glands of the pancreas and the intestine (Perez-Casanova et al. 2006), and its development is considered to represent the last step in the maturation of the digestive system in fish (Zambonino Infante and Cahu 2001). NAK encodes the subunit alpha of Na^+–K^+ ATPase, which is a key enzyme in fish osmoregulation. This protein is highly abundant in fish and expressed in osmoregulatory organs, including the intestines (Tseng et al. 2013, Wilson et al. 2002). Thus, the observation that NAK and TRP1α expression did not significantly change from 32 dph to 46 dph in an hypercapnic environment may reflect deficiencies or a lag in the development of the digestive system. This in turn could be the consequence of a prolonged stress response during metamorphosis.

However, while these findings propose an influence of carbon dioxide on developmental dynamics, results have to be considered with care. A relatively high variance in the data, which may be another side effect of RNA extraction from whole larvae, as well as the restriction to only two distinct sampling points, could mean that certain findings, despite a statistical significance, have no further biological relevance. Therefore, this lead demands for more scientific research to confirm and, eventually, elucidate details of this potential carryover effect.

Implications for cohort recruitment

Despite evidence which suggests that individuals suffering from severe pathologies died before the second sampling event (see Stiasny et al. 2016), the possibility that cod larvae were able to recover until the age of 46 dph cannot entirely be dismissed. Schiffer et al. (2013) demonstrated that energy demanding responses to OA during certain vulnerable stages in crustacean larvae (*Hyas araneus*) can indeed lead towards successful acclimatization. Thus, in the forecasted acidification of the oceans, a gradual decrease in pH may allow Atlantic cod larvae to adapt in the long run. The observed variance within groups in the exhibition of pathologies as well as in the transcriptional adjustment, may allow selection to act. In this regard, our data confirms that around 32 dph cod are at a developmental stage characterized by increased vulnerability to disturbances of the acid-base equilibrium. However, even if a fraction of a cohort is able to compensate maladaptive effects during such a period, there remains the possibility that this may comprise the animals at an older age or is accomplished at the expense of overall life-time fitness (Pechnik 2006).

On the other hand, it has been shown, that chronic acid-base disturbances in teleosts negatively impact ventilation rates, blood pressure and heart rates, which ultimately can result in death (Esbaugh et al. 2012). Therefore, assuming oxidative stress and impaired capacity as a function of stress intensity, we speculate that larvae which were able to up-regulate the expression of genes associated with glycogen and fatty acid synthesis were also able to cope with OA, while individuals who failed to trigger this part of the stress response may have died between the two sampling points. Following from that supposition, the pattern detected in cod larvae subjected to the chronic exposure to 1800 µatm pCO_2 could be of special interest as it may link to sub-lethal stress, hence, advocating gys1, 6PGL and the ratio of AcoA to FAS as potential biomarkers for the distinction between successful and failed stress responses.

Conclusions

Most of the connections deduced from our data were based on studies conducted in mammalian models or isolated cells. Accordingly, more scientific research is required to validate and unravel further details of the processes which

translate the tested conditions into the respective stress-induced phenotypes (cellular and physiological). Since our findings suggested tissue specific effects of hypercapnia, we advise to avoid whole larvae samples in future studies, if possible. This approach would likely provide a more distinct and nuanced gene expression pattern.

However, our study demonstrated noteworthy changes in the expression of metabolism-associated genes in cod larvae exposed to environmental hypercapnia at 32 dph. In particular, signs of increased fatty acid and glycogen metabolism were in line with previously reported pathologies such as random lipid accumulation. Enhanced expression of HIF-1 regulated gys1 also supports the assumption of local shortcomings in oxygen provision. Further, expression of genes such as PPARβ and 6PGL hinted at oxidative stress, which, in non-adipocytes, can be fostered by an overabundance of lipids as well as hypercapnia itself. In agreement with Kassahn's model of capacity limitation, increasing oxidative stress with increasing stress intensity could provide an explanation for both, a dose-dependent transcriptional adjustment to hypercapnia as well as common elements of the stress response.

Acknowledgments

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Discussion

In order to gain new insights into the transcriptional response of fish to varying intensities of chronic stress, I investigated the impact of dietary antinutritional factors on the liver and severe environmental hypercapnia on the gills of juvenile turbot (*Psetta maxima*, Chapters I & II), as well as ocean acidification on whole cod larvae (*Gadus morhua*, Chapter III). Across studies, each treatment level resulted at least in one gene with significant changes in transcript abundances, thus, confirming the necessity of transcriptional adjustments in the face of persistent stress. Furthermore, the inclusion of histological and physiological data provided additional information, which helped to distinguish between adjustment processes with potential negative effects on the cellular performance capacity (Chapters I & II) and clear indications of a metabolic dysfunction (chapter III). Vice versa, the lack of pathogenic symptoms in turbot liver and gills after the exposure to their respective tested stressor also illustrated the great advantage of molecular data, as gene expression analyses were able to reveal elements of the stress response which otherwise would have remained undiscovered.

In this regard, a few overarching themes emerged: First, chronic stress, even when mild, seems to trigger dose-specific adjustments in the transcription machinery. This was indicated by the expression profiles of individual genes (for example LMP7 vs. HSP70 in chapter I) as well as by between class analyses (BCAs). The latter, which were employed to detect differences in the overall expression patterns of selected target genes for juvenile turbot, demonstrated differing adjustment strategies - in particular between treatment levels (Chapters I & II). Second, the implications regarding the modulations of cellular biochemistry inferred from the single studies, as well as their contemplation in context with each other, suggested that the cellular stress response comprises a) elevated concentrations of reactive oxygen and nitrogen species (ROS and RNS, respectively), b) hypoxia or hypoxia-like effects, c) shifts in the lipid- and fatty acid metabolism and d) alterations in metabolic activity and protein synthesis capacities.

Yet, while the entirety of responsive genes in the second chapter provided strong and coherent indications for these effects, in particular regarding the involvement of ROS, results obtained in chapter III were vague and more difficult to conciliate. Here, opaque dynamics between the two sampled time points, as well as

the extraction of RNA from whole larvae samples, which may have blended the expression profiles of individual tissue types, complicated the interpretation of the results.

Nevertheless, the above mentioned putative effects on the cellular milieu are not only interconnected, but also bear the potential to reinforce each other. In the following sections, I will elaborate on further details and on how amplifying mechanisms may become more and more relevant as stress intensity increases. Furthermore, the ambivalent nature of metabolic end products, such as ROS, provide a good explanation for a dose-dependent divergent regulation of gene expression, as the ratio between beneficial regulatory and detrimental disruptive effects is set by their intracellular concentrations. In addition, the perpetual activity of such substances above a baseline level may further explain how chronic stress can entail negative cellular and physiological effects.

Chronic stress-induced changes in cellular biochemistry

The relevance of reactive oxygen species was especially emphasised in the second chapter by the up-regulation of genes linked to antioxidant function (e.g. Grim19, PRDX), activity of redox-sensitive transcription factors (e.g. Akirin, LMP7) or direct ROS formation (e.g. IL-1ß). Also, assumed lipotoxicity in hypercapnia-exposed cod larvae (chapter III) could foster oxidative stress (Egnatchik et al. 2014). Hence, my data seems to confirm the notion postulated by Kültz (2005, 2003), in that most stressors, despite different modes of actions, converge on elevated concentrations of reactive molecule species. Furthermore, my results demonstrated, that ROS- and RNS- (which can be summarized as reactive molecule species, RMS) driven changes in gene expression may also be a relevant way beyond the initial phase of the stress response. These molecules cause random oxidation processes, which impair the functionality of enzymes and other cellular components, which in turn provide the cell with information about its integrity. However, these molecules also affect gene expression by influencing the activity of several redox-sensitive transcription factors, such as AMP-activated protein kinase (AMPK), nuclear factor-kappa B (NF-κB) and Activator protein 1 (AP-1) (Xu et al. 2012, Hamanaka & Chandel 2010, Giordano 2005, Karin et al. 2001).

While it is likely that a combination of different substances with a high oxidation potential accumulate during stress, the dominant class of these reactive molecule species seems to be ROS. Indeed, indications of oxidative stress in aquatic animals have also been found in response to pollutants or changes in crucial environmental factors, such as temperature, salinity and oxygen (Lushchak 2011). ROS are constantly generated in small concentrations within the mitochondria as electrons occasionally leak into the matrix and react with oxygen to form superoxide, which in turn can spark subsequent ROS producing reactions (Lushchak 2011, Kuznetsov et al. 2011, Hamanaka & Chandel 2010). Thus, enhanced energy demand and, therefore, elevated activity of the mitochondrial electron transport chain (ETC), is considered the main source for stress induced increase in reactive molecule species. In addition, so called endoplasmic reticulum stress (ER stress) and associated reallocation of calcium (Han & Kaufman 2016, Zhang & Kaufman 2008, Malhotra & Kaufman 2007), as well as the up-regulation of certain oxidases (for example NADPH Oxidase, Malhotra & Kaufman 2007) provide additional stress-related mechanisms by which these molecules can increase in abundance.

Furthermore, environmental hypercapnia, which was applied as the main stressor in the chapters II & III, can directly add to the generation of reactive molecule species: Carbon dioxide is known to react with peroxynitrite, an offspring of the mitochondria-borne superoxide pathway, which forms further oxidative and nitrosative substances (Dean 2010, see also chapters II & III). While respective ties are more complex and less straight forward, differences in dietary composition (as tested in chapter I) may also influence the redox-balance of the cell. Potentially, varying amounts of antioxidants, antinutritional factors and other compounds can influence abundance and activity of receptors, transcription factors and other important regulatory elements, which may ultimately affect the cell's redox-status. For example, Olsvik et al. (2011) found evidence for altered hepatic gene expression and antioxidative defence in Atlantic Salmon (*Salmo salar*), dependent on the administered experimental diet. Regardless of the source, oxidative stress arises when antioxidant mechanisms of the cell are no longer able to retain ROS within the borders of a regulatory imbalance and detrimental random oxidation processes start to outweigh desired regulatory effects (Dröge 2002).

Overall, reactive molecule species as central mediators of the chronic stress response may also provide an explanation for both, the cellular stress response and

the cellular homeostatic response (see Kültz 2005). While the CSR could be regarded as a response to altered redox-states and augmenting cellular damage in general, the CHR may be triggered by a situation dependent RMS profile, as transcription factors and other regulatory elements differ in their sensitivity to different oxidative agents (Thorpe et al. 2004). This hypothesis is supported by a genome-wide transcriptional analysis in yeast, which tested different stressors and found convincing evidence for ROS profile dependent changes in gene expression, in addition to a commonly activated palette of genes (Temple et al. 2005). Consequently, not only RMS concentration, but also composition of the RMS profile may contribute to the development of a situation-dependent phenotype.

Shifts in general lipid and fatty acid metabolism, which were demonstrated by changes in the mRNA concentrations of DFAD (Chapters I and II), apolipoprotein E (Chapter II), as well as AcoA, FAS and, in co-dependence of progressing larval development, CPT1a and PPARβ (Chapter III), may also be connected to stress-induced increases in intracellular ROS concentrations. However, as indicated by the functional differences of the affected genes, this link may be of complex nature and dependent on multiple factors. Yet, the different studies exemplified that especially stress intensity (and therefore concentration of intracellular RMS) may determine whether alterations in the mRNA abundances of genes associated with lipid metabolism rather reflect a compensatory response or a derailed lipid homeostasis.

The results presented in chapter III potentially represent the latter case: Here, lipotoxicity, a phenomenon in which excess lipids cause maladaptive effects (Han & Kaufman 2016, Brookheart et at. 2009), was discussed as a potential contributor to observed gene expression patterns. This phenomenon is known to provoke oxidative stress by promoting ER stress. Symptoms, which are comparable to those reported for cod larvae, for example dyslipidemia or steatosis, are also known to develop as a possible consequence of ER stress (Basseri & Austin 2012). Within the mammalian model, elevated reactive oxygen species in hepatitis c virus (HCV) infected hepatoma cells have been demonstrated to modulate fatty acid metabolism (Douglas et al. 2016). This occurred together with growth arrest and not only resulted in a significant increase in fatty acids, especially in PUFAs, but also in an accumulation of lipids in general.

In addition to potential malfunctions in pathways associated with lipogenesis and -lysis, oxidative and nitrosative stress also degrades the integrity of membranes

and other lipid structures. In particular PUFAs are susceptible to oxidation (Avery 2011) which in turn spawns numerous subsequent symptoms as these molecules are not only essential for the proper function of organelles but also exert regulatory tasks themselves (Tocher 2003). Thus, enhanced fatty acid synthesis, as indicated in the chapters I & II may rather reflect a compensatory response, as the cell aims to compensate for losses induced by increasing RMS concentrations.

The idea, that reactive oxygen species are central drivers of the stress response, was further incorporated in the model of oxygen- and capacity limitation (Kassahn et al. 2009, for more details see Chapter II). This model also described a general, concomitant decrease in oxygen availability, dependent on the intensity of the stressor. Anaemia, which can arise in fish because of various environmental stressors, including malnutrition, may provide a systemic cause for reduced oxygen availability (Witeska 2015). As a matter of fact, signs of hypoxia-like effects were noted in chapter III, with the up-regulation of HIF-1 associated glycogen synthase I. Diminished capability to utilize oxygen in the cells or ROS induced activation of hypoxia-associated pathways may also link to reduced energy availability (indicated by the down-regulation of IGF-1 and transferrin in Chapter I), reduced metabolic activity and suppressed protein synthesis (indicated by the down-regulation of COX, STAT2 and EF1a in Chapter II) or both (indicated by a supposed inertia of the stress response in the highest tested treatment level in Chapter III). This connection is also supported by a previous study, which tested the effects of hypoxia on juvenile gilthead sea bream (*Sparus aurata*) and found extensive reductions in almost all genes associated with the respiratory chain in the blood cells, resulting in diminished work capacities (Martos-Sitcha et al. 2017). However, in addition to diminished rates of cellular respiration, suppressed protein synthesis can also directly arise as a consequence of enhanced RMS activity since these substances are able to interfere with transcription and translation (Avery 2011).

Stress-intensity depended transcriptional adjustments to chronic stress

It can be assumed that several maladaptive consequences of elevated reactive molecule species, for example a disrupted lipid homeostasis, entail further deleterious effects. Thus, alongside an increasing stress intensity, the impact of oxidative (and nitrosative) stress becomes amplified. The joint interactions of

intracellular reactive molecule species, antioxidants and, if uncompensated, resultant disruptions of cellular homeostasis may define hallmarks, beyond which significant modulations of signalling cascades provide a foundation for divergent, dose-dependent adjustments of the transcription machinery. Given the possibility that chronic stress may lead to an alleviation or, in some cases, even an abolishment of cortisol mediated regulation of gene expression (see introduction), these mechanisms may dominate the cellular interior and, therefore, provide the best explanation for the recurring theme of an intensity dependent overall modulation of gene expression within my studies.

Due to their huge impact on the cellular biochemistry, certain organelles may have key roles in the translation and amplification of external stress signals into extensive cellular effects. Special consideration must be directed towards the mitochondria as the primary side of ROS production and, therefore, as the main target of these molecules (Ott et al. 2007). Due to their vital importance in the generation of energy and general metabolism, dysfunctions pose severe threats to cellular homeostasis. Disruption of the electron transport chain (ETC) or the membranes further enhances the production of reactive molecule species, as well as their release into the cytosol. Impaired integrity of the mitochondria could also be linked to the other biochemical changes suggested by my data. Suppressed protein synthesis, for example, may be a direct consequence of hampered mitochondrial functionality. Alternatively, diminished capacity for biosynthesis and other functions may also be lowered by uncoupling of the ETC from the proton motive force, which is considered a protective measure, aiming to reduce the amount of mitochondria-borne ROS (Brand et al. 2004).

The endoplasmic reticulum is another cell compartment which may heavily contribute to potential dose-dependent transcriptional adjustments in the response to chronic stress (Han & Kaufman 2016, Zhang & Kaufman 2008). Due to close proximity to the mitochondria, a tight cross-talk between these organelles can significantly foster the dissemination and amplification of stress signals. For example, ER-stress induced release of calcium ions is considered to depolarize the inner mitochondrial membrane (Zhang & Kaufman 2008). The resultant disturbances of the ETC cause an increase in reactive oxygen species. ROS, in turn, can provoke Ca^{2+} release from the ER (Csordás & Hajnóczky 2010, Yan et al. 2008). In particular, different sensibilities of ER-associated enzymes to oxidizing agents could provide a

basis for a "dose-dependent order of activation" (Csordás & Hajnóczky 2010). In case of stress intensities below the apoptosis inducing threshold, this in turn could benefit the establishment of distinct gene expression patterns.

Implications for allostasis and hormesis

The transient nature of a ROS/RNS mediated cellular stress response and all its subsequent consequences, especially with respect to mitochondrial integrity, may also proof to be a useful extension for current stress models. Allostasis, for example, emphasizes the condition of the whole organism, which made it difficult to apply this framework to the cellular level. Previous attempts to incorporate the cellular stress response into the allostatic model focused on mitochondria as the essential linchpins of energy metabolism and cellular signalling: A recent publication suggested "mitochondrial allostatic load" in dependency of glucose concentrations (Picard et al. 2014).

However, the inclusion of reactive molecule species into the allostatic framework may provide an even better indication regarding the condition of the mitochondria, as well as the cell as a whole. Moreover, the link between stress intensity and RMS concentrations could provide a mechanistic explanation for allostatic load and overload. Allostatic load and overload may then trace back to a regulatory imbalance and oxidative/nitrosative stress, respectively (see Dröge 2002). At the same time, the persistent upkeep of elevated RMS concentrations during chronic stress would maintain a constant activity of redox-sensitive transcription factors, thus, further facilitating an explanation for the manifestation of wear and tear effects. AMPK, for example, while apparently involved in the regulation of antioxidants is also capable to promote apoptosis via caspase activation (Douglas et al. 2016).

RMS mediated adjustments may also provide a valuable addition to hormesis. The enhanced resistance against increasing stress intensities or other stressors after the exposure to mild stress may originate on a cellular level and might be caused by an overcompensation of a small regulatory imbalance. Potentially, an "all-or-nothing"-type of response may result in an over-expression of several antioxidants and other compensatory substances, generating an extra capacity to deal with subsequent challenges.

Implications for animal welfare

Under the premise that stress-induced damages within cells translate into maladaptive repercussions on higher levels of biological organisation, the notion that reactive molecule species are highly relevant for the chronic CSR could also enhance functional approaches to animal welfare. Thus, in the light of my results and the implications deviated from them, I hypothesize that good animal welfare can be assumed, if environmental conditions provided for a fish species do not inflict oxidative or nitrosative stress within the cells of a certain organ or tissue type, hence, maintaining its overall functionality.

Consequently, this supposition may proof especially useful, when physiological or histological data is inconclusive. For example, despite an apparent inviolacy, evidence for suppressed protein synthesis and metabolic activity in gills dissected from turbot subjected to the highest tested level of carbon dioxide (Chapter II) clearly suggest reduced capacity to cope with the stressor, as well as with additional challenges. These signs of reduced capacity to maintain homeostasis not only indicate diminished welfare but may also mark the onset of pending pathologies.

While the derivation of welfare penalties under conditions of oxidative or nitrosative stress is relatively straight forward, the incessant nature of chronic stress makes matters more complicated: As already mentioned earlier, the constant perpetuation of redox-sensitive transcription factors may provoke deleterious aftermath even below the line of oxidative or nitrosative stress. In addition to wear and tear effects, their prolonged activity may also entail morphological and functional changes of the cell. Such changes could also include alterations in the composition or abundance of membrane-bound receptors, which in turn could provide an explanation for a possible advancing inertness of the cell to endocrine control. This is relevant, as the ability to commence and cease a stress response suitable to the imposed challenge has been identified as a vital element of animal welfare (Korte et al. 2007). At the same time, mild stress in the hormetic range does not seem to have any downsides, though this phenomenon is not well described for enduring stress (Calabrese and Baldwin, 2002). In fact, the data presented in chapter I could reflect exactly these two potential outcomes of a tolerable and persistent challenge not exhausting antioxidant capacities. On the one hand, hormetic mechanisms persist and the morphology of the cell remains unchanged (R33), while on the other hand certain

regulatory variations culminate in shifting receptor abundances, which in turn makes the cell inflexible towards future changes in the environment (R66). In the latter case, the data could imply a potential reduction of transferrin and growth hormone receptors in liver cells. If this assumption can be validated, it would provide mechanism by which reduced growth rates could be linked to eased hormonal control of the intracellular milieu and, thus, support the current view that a decline in performance parameters can be regarded as impaired fish welfare (Segner et al. 2012). However, at this point, a correlation between reactive molecule species and the cell's ability to communicate with its exterior requires much more scientific validation. A deeper look into the relationship between stress intensity and cellular receptor composition may be an interesting and rewarding target for future research effort.

Biomarkers for chronic stress research

Based on my findings, several of the applied GOIs may be valuable biomarkers for both, the detection of identified effects putatively caused by chronic stress, as well as the confirmation of these effects in different settings. The most promising genes are those yielding altered mRNA concentrations across different studies, namely COX and LMP7 (Chapters I & II, respectively). Observed changes in gene expression support the idea that COX expression levels may be positively correlated with metabolic activity. While it is likely that there is also a tie to the mitochondrial integrity in general, the connection is still vague and needs further evaluation. The latter would be of special interest, when attempting to apply COX as an indicator for intracellular RMS concentrations. LMP7, on the other hand, may indicate proteasome activity and, therefore, can be useful within studies that aim to further investigate protein metabolism and protein quality control during chronic stress.

It is therefore interesting to note that all tested members of the heat shock protein family, as representatives of an alternative strategy to maintain protein integrity, appeared to have no to little relevance under the tested conditions. The only exception was seen in chapter I, where HSP70 and LMP7 displayed oppositional dynamics. In terms of chronic stress, these outcomes challenge the general validity of HSP transcripts as biomarkers, at least when applying gene expression analysis (Gupta et al. 2010, Feder & Hofmann 1999). Factors, such as stress intensity, may have to be

taken into account. Consequently, to test whether different stress intensities redound to different strategies for the insurance of protein quality, future studies would benefit from including heat shock proteins when applying LMP7, and vice versa.

Further, strong evidence of pronounced stress-induced alterations in the lipid and fatty acid metabolism requires further study. The observation, that a disrupted lipid homeostasis seems to pose a crucial turning point towards the development of severe pathologies calls for related pathways to be primary targets. In particular, fatty acid synthesis and the role of PUFAs require a deepening examination. Thus, due to highly significant changes in different tissues of the turbot, I strongly recommend including DFAD in respective target gene approaches. This is also true for glycogen synthase isoforms, which may not only be connected to the lipid homeostasis but may also help to advance knowledge regarding the role of oxygen availability, as well as of transcription factors, such as HIF-1.

Finally, IGF-1 and, to some extent, transferrin should be subjected to further scrutiny, as these genes could provide valuable information regarding energy allocation processes and overall capacity of the cell. If this assumption can be validated, expression analysis of these genes may constitute a valuable surrogate measurement when attempting to assess the animal's condition (i.e. allostatic state, see introduction). In addition, derived from this hypothesis, these genes may also allow to deduce conclusions concerning the individual's susceptibility to stress in general.

Future studies may benefit from the inclusion of genes which are associated with reactive molecule species (antioxidants or regulated by redox-sensitive transcription factors) or any of the biochemical adjustments implied by my data. This is in particular true for genes of the mitochondria or the fatty acid metabolism. In the light of current technological advances, an efficient approach could utilize large-scale RNA-sequencing approaches as an initial step to identify potential target genes, followed by RT-qPCR in which the expression behaviour of these genes is examined.

Conclusion

Gene expression analysis is a valuable method to obtain new insights regarding the chronic, sub-lethal cellular stress response in teleosts. However, due to the pleiotropic nature of many genes, as well as potential incongruencies between

transcription and translation (e.g. Liu & Qian 2014), the inclusion of physiological and histological information helped to provide context and is strongly recommended for future studies. Furthermore, it is important to bear in mind that my conclusions are derived from multiple leads, rather than from one or two striking finds. While providing a relatively coherent explanation for observed gene expression pattern, more research is required to confirm and validate my take on the results. In particular the role of both, reactive molecule species and changes in the fatty acid metabolism during chronic stress require further investigation. Thereby, the impact of RMS on the cell's ability to communicate with its surrounding, as well as the relevance of the RMS composition on the manifestation of a certain situation-dependent phenotype may be specifically interesting.

Nevertheless, the indications derived from my results also contribute to the concepts of allostasis and hormesis. The suggested link between stress intensity, intracellular RMS concentrations and their increasingly negative impact on the cellular milieu may provide a functional explanation for allostatic load and overload. In the low-dose range, an activation of excess anti-oxidants may explain hormetic effects. Consequently, information regarding a stressor's ability to inflict oxidative stress in the cells of vital tissues could be tested and used as a metric to infer whether functional welfare standards are met or violated. In this regard, some of the target genes applied in my experiments bear the potential to provide reliable information regarding the cells condition and, therefore, could be used as stress biomarkers. Still, the utility of genes, such as COX, DFAD or glycogen synthase, has to be tested in additional studies, which also comprise different species, tissues and stressors.

A major goal of my studies was to identify commonalities in the chronic CSR. Despite different stressors, species or tissues, my results repeatedly indicated stress intensity-dependent transcriptional adjustments. The collective consideration of available data suggested that these patterns are mediated by an interconnected array of processes, in which the generation of reactive molecule species from different sources may occupy a central role. Thus, while RMS (in particular ROS) have long been considered an essential element of the CSR during the acute phase, my results strongly imply their relevancy for chronic stress, as well. Due to potential biochemical and morphological alterations in the cell, RMS may even become the main driving force behind regulatory mechanisms. Thereby, the constant up-keep of redox-sensitive transcription factors may further provide an explanation on how even mild

chronic stress, in which anti-oxidants are still able to prevent random oxidation events, can evoke wear and tear like effects.

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List of tables

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Description of the individual scientific contribution to multiple-author publications

The chapters presented in this thesis are published in peer-reviewed scientific journals (Chapter I and II) or have been prepared for a submission (Chapter III). The contributions of each author are listed below:

Chapter I:

Effects of dietary purified rapeseed protein concentrate on hepatic gene expression in juvenile turbot (*Psetta maxima*)
Authors: B. T. Hermann, Thorsten B. H. Reusch and R. Hanel.
Published in **Aquaculture Nutrition** 22, 170–180 (2016). doi.org/10.1111/anu.12251

Contributions: BTH conceptualised and conducted the gene expression analysis, analysed the data and wrote the manuscript. All authors discussed the results and contributed to the final manuscript. The tissue samples were provided by Hanno Slawski (excluded from authors list by own request) who designed and conducted the original experiment.

Chapter II:

Divergent gene expression in the gills of juvenile turbot (*Psetta maxima*) exposed to chronic severe hypercapnia indicates dose-dependent increase in intracellular oxidative stress and hypoxia.
Authors: B. T. Hermann, K.T. Stiller, S. Würtz, K. H. Vanselow, C. Schulz
Published in **Aquatic Toxicology** 206, 72-80 (2019).
doi.org/10.1016/j.aquatox.2018.10.023

Contributions: KTS, KHV and CS designed the experiment and KTS carried out the experiment. SW performed histological analysis of the gills. BTH conceptualized and conducted the gene expression analysis, analysed the data and wrote the manuscript with input and final approval from all authors.

Chapter III:

Differential gene expression in Atlantic cod larvae (*Gadus morhua*) under future ocean acidification.
Authors: B.T.Hermann, A.Y. Frommel, K. Michael, M. Lucassen, R. Hanel, T.B.H. Reusch
Manuscript prepared for submission.

Contributions: AYF designed and conducted the experiment. BTH, AYF and KM conceptualized and conducted the gene expression analysis. Data analysis was done by BTH. Manuscript was written by BTH with contributions from AYF. All authors provided critical feedback and helped shaping the analysis and manuscript.

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Eidesstattliche Erklärung

Hiermit versichere ich, dass diese Dissertation, abgesehen von der Beratung durch meinen Betreuer, selbständig von mir angefertigt wurde und dass sie nach Inhalt und Form meine eigene Arbeit ist. Ich habe keine als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt. Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren. Das erste Kapitel dieser Dissertation wurde in einer international anerkannten Fachzeitschrift, *Aquaculture Nutrition*, veröffentlicht (zusammen mit Thorsten B.H. Reusch und Reinhold Hanel als Co-Autoren).

Berlin, den 24. September 2018 Bernd Timo Hermann